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13. ABSTRACT *(Maximum 200*

The goal of this program is to develop and test a fibrin based tissue adhesive and wound dressing system specifically to be usable on the battlefield. The tissue adhesive is a fibrin monomer based powder which is supplied in two forms: included within the pores of an open cell hydrophilic polyurethane foam intended for controlling deep tissue bleeding; or on the surface of an adhesive backed silicone film designed for treating superficial injury. In this contract period, the analytic characterization of the hemostatic agent was completed, and manufacturing methods for producing the agent have been developed. Animal experiments to evaluate its effect on final wound healing were also started. The final formulation for the hydrophilic foam delivery system was developed, and this material passed all USP Class VI toxicological tests. Testing of the silicone film wound dressing was begun, including an evaluation of timed release antibiotics incorporated into the film. U.S. patent 5,464,471 has been awarded, which covers the tissue adhesive itself and both delivery systems.

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R. L. W

Robert L. Whalen, Ph.D.
April 10, 1996

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Introduction

In this program, our goal is to develop and test a fibrin based tissue adhesive and wound dressing system specifically to be usable on the battlefield. To be employable under these conditions, we believe the tissue adhesive should be usable in a dry form and not require the pre-mixing of ingredients. It should also incorporate a delivery system which protects and seals the site of injury from contamination.

Work conducted during year one of this Phase II program has yielded a number of important results. A majority of the key tasks in the program have been successfully completed, and no significant problems have yet been identified. U.S. Patent Number 5,464,471, which covers the basic formulation of the new tissue adhesive and both delivery systems currently under development, was awarded on November 15, 1995. A copy of that patent is included in Appendix A of this report.

In Task I, Formulation Development, the analytical characterization of the best formulation for the hemostatic agent was completed, including the development of a profile of the complete formulation for quality control purposes, using gel permeation chromatography. A final process for manufacturing the required fibrin monomer was also established. The appropriate method of sterilization to be employed and its impact on this manufacturing process was also addressed. These results and the methodology employed are summarized in this report.

In Task II, chronic Tissue Healing Studies, animal experiments to study effects of the tissue adhesive on wound healing were begun using a splenic injury model. These experiments are being conducted at the Cleveland Clinic Foundation (Cleveland, OH), and they were begun late in the most recent quarter.

As part of Task III, Delivery System Development and Testing, the development of the hydrophilic polyurethane foam carrier for the tissue adhesive has been completed, thus providing a delivery system to help control bleeding with deep tissue injury. The hemostatic agent is loaded directly into the open cells of this foam-like material, which is designed to be compressed into the site of deep tissue injuries with the goal of achieving temporary hemostasis of potential arterial bleeding until surgical intervention is possible. The foam thus provides a vehicle for getting the tissue adhesive to the site of bleeding and mechanical compression to aid in controlling this type of hemorrhage.

The development and testing of the antibiotic film delivery system intended for treating superficial injury also continued during the most recent quarter. A number of soft silicone cured films have been selected with testing running concurrently. Films have been manufactured from the

initial formulations, and the subsequent drug elution rates determined by HPLC. Having focused on optimization of the drug release profile, drug elution rates and microbiological activity have been identified with the results provided under Task IV, Controlled Release of Antibiotics

Finally, in Task V, Packaging, Shelf Life, and Toxicity Evaluations, toxicity testing on the foam carrier was successfully completed (USP Class VI), and these results are outlined in the body of this report. Tests to determine the effects of various methods of sterilization on the foam delivery system were also carried out. It seems clear that heat sterilization is deleterious to the foam material; however, any polyol (from which the foam material is made) which might be released, is not highly toxic.

While our primary focus in this program is on the military applications of the new hemostatic agent/tissue adhesive system, it is obvious that the civilian need for this type of product is also significant. There is a definite need for improved hemostatic agents during emergency first aid in the pre-hospital setting or even in routine emergency room practice itself. Therefore, this is technology which has both important military and civilian uses, as well as significant commercial potential.

We believe the regulatory issues which have prevented the approval of fibrin tissue adhesives in this country also are soon to be addressable. Recombinant human fibrinogen is likely to become commercially available in the near future. Since our new agent will then utilize no components derived directly from pooled human donor blood, the issue of disease transmission via the product is largely circumvented. Regulatory approval of this product should thus be facilitated.

The combined wound dressing/tissue adhesive approach we are developing would appear to be a readily commercialized product for use by EMT's in the pre-hospital setting. The clinical trials necessary to obtain FDA approval to market such a product in this country are beyond the scope of this SBIR effort, but the SBIR achievements must be sufficient to justify continued investment in the product from private sources.

Task I. Formulation Development

In this task our objectives are to develop the most suitable formulation for the tissue adhesive, characterize analytically the complete formulation, and establish manufacturing and sterilization techniques which will result in consistent production of the material. Utilizing gel permeation chromatography, we completed the characterization of the best formulation, developed a process for obtaining fibrin monomer to use in the tissue adhesive, and established the appropriate sterilization methods.

Process Development and Sterilization of the Fibrin Monomer

Outlined below are the steps which led to the identification and qauntification of the fibrin monomer compound utilizing HPLC analysis.

In order to develop and quantify a process for the development and sterilization of the fibrin monomer used in the tissue adhesive, it became necessary to identify and quantify the constituents in the preparation solution to ensure accurate production of the agent. Techniques to detect such plasma products have evolved with the development of highly sensitive degradation methods for protein sequence analysis. These microscale sequencing techniques demanded for equally sensitive techniques for purification, isolation, fragmentation and identification. For all of these purposes, high performance liquid chromatography (HPLC) has proved to be an excellent tool.

A process for preparing the fibrin monomer has been defined and reported in the last quarterly report. The hemostatic agent consists of a lyophilized solution of fibrin monomer, aprotinin, thrombin, and calcium chloride.

We have examined the effects of adding aprotinin to the formulation and have determined that its addition to the formulation is of no benefit acutely, but the results from chronic tissue healing studies are not yet available. Bovine aprotinin (Trasylol,® Bayer Corporation, West Haven, CT) was obtained in a 10,000 KIU/ml concentration. When added to the hemostatic agent, it is used in the amount of 5,000 KIU. To study its effect on acute hemostasis, we conducted in vitro measurements using a modified activated clotting time test similar to the measurements made in Phase I. The results of these tests indicated that the addition of aprotinin to the fibrin formulation made no measurable difference.

HPLC Analysis and Molecular Weight Determination

A 0.05% solution of the lyophilized fibrin monomer was prepared in buffered saline. The sample was run using a Water's 510 isocratic pump at 1 ml per minute using buffered saline as the mobile phase. Size exclusion chromatography was performed using a Shodex 8mmx300mm 7um KW-803 column using Water's 450 uv detector; detection was performed at a setting of 280 nm.

In order to determine the molecular weight range of the fibrin monomer a calibration curve was prepared using water soluble proteins having known molecular weights. Standard 0.05% concentrated solutions

of Blue Dextrin (2000K), g-Globulin (150K), Bovine Serum Albumin (66.3K) and Myoglobin (17.6K).

Figure 1 shows the calibration curve generated using these standards. The relative molecular weight was single modal and determined to be 346K Daltons when prepared using 250 units of thrombin to 100 mgs of fibrinogen. When 125 units of thrombin was used to prepare the fibrin monomer bimodal molecular weights were evident. These were estimated to be 350K and 316K. The former comprised approximately 65% of the sample and the remaining 35% was attributable to the 316K molecular weight fraction.

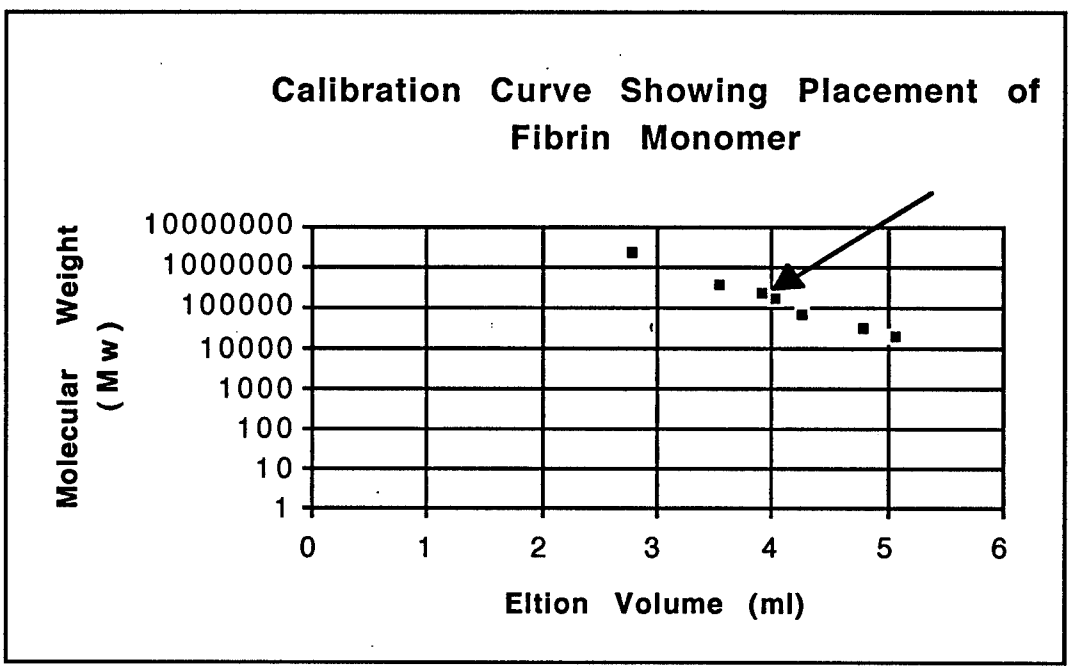


Figure 1. Molecular weight calibration curve.

Further characterization was required to determine whether thrombin concentration played a role in the integrity of the formed fibrin clot. In order to examine these effects the units of thrombin were varied from 1, 10, 50, and 500.

Fibrin monomer was prepared by mixing stochiometric amounts of fibrinogen and thrombin (in the experimentally varied amounts), the subsequent solution was then mixed utilizing a gentle shaking motion. The soft gel that formed was dissolved using a 10% ammonium hydroxide solution. The soft gel was placed in a bell jar and a vacuum applied to remove the ammonia gas allowing the remaining solution to be frozen. The

frozen solution was placed on the lyophilizer and dried. The white powder that was formed was visually inspected and then a sample removed for HPLC analysis.

The first formulation was made utilizing 1 unit of thrombin. The 1 unit of thrombin was added to the fibrin solution. The solution was mixed for 3 hours with a clot being allowed to form. Then, as according to protocol the resultant clot was rinsed with 10 mls of normal saline solution. The fibrin-thrombin clot was then frozen and placed on the lyophilizer for 12 hours. The freeze dried clot was then removed and weighed using a Mettler balance. The percent yield after drying was determined to be 79.6%

The second formulation was made utilizing 10 units of thrombin. The fibrin solution was reconstituted according to protocol with the 10 units of thrombin added at the proper time. The solution was then mixed for 3 hours at which time the resultant clot was rinsed with 10 mls of normal saline solution. The fibrin-thrombin clot was then frozen and placed on the lyophylizer for 12 hours.

The freeze dried clot was then removed and weighed using a Mettler balance. The percent yield after drying was determined to be 115%. The dried clot was then placed in a filter funnel and distilled water was then added. The clot was filtered through a 10 micron filter and then vacuum dried. The percent yield after drying was 80.1%

The third formulation was made utilizing only 50 units of thrombin. The 50 units of thrombin were added to the fibrinogen solution. The solution was mixed for 3 hours with a clot being allowed to form. Then, as according to protocol the resultant clot was rinsed with 10 mls of normal saline solution. The fibrin-thrombin clot was then frozen and placed on the lyophilizer for 12 hours.

The freeze dried clot was then removed and weighed using a Mettler balance. Then, as indicated above the clot was placed in the freeze dryer followed by placement in the lyophilizer for 12 hours. The freeze dried clot was then removed and weighed. Rinsed with 10 mls distilled water followed by addition of distilled water. The percent yield after drying was 89.6%

The fourth formulation was made with 500 units of thrombin. This particular formulation, when added to the predetermined fibrin solution, gelled almost immediately. The solution was allowed to mix via automatic mixer for 1 hour. Distilled water was then added to the preformed clot, followed by a distilled water rinse. Then, as indicated above,the clot was placed in the freeze dryer followed by placement in the lyophilizer for 12 hours. The freeze dried clot was then removed and weighed. The percent

yield was 95%. The dried clot was then placed in a filter funnel and rinsed with water and later dried. The percent yield after drying was 75.6%

Shown in Figure 2 on the following page is a graphical analysis showing the results of the various thrombin formulations and their relative effect in regards to the amount of clottable protein.

The molecular weight and composition of the fibrin monomer for the four formulations were determined to be as follows: the 500 unit formulation was composed of 45% 288K and 55% 129k; the 50 unit of 24% 249K and 76% 82K; the 10 unit of 100% 277K; and the 1 unit formulation was 100% 305K.

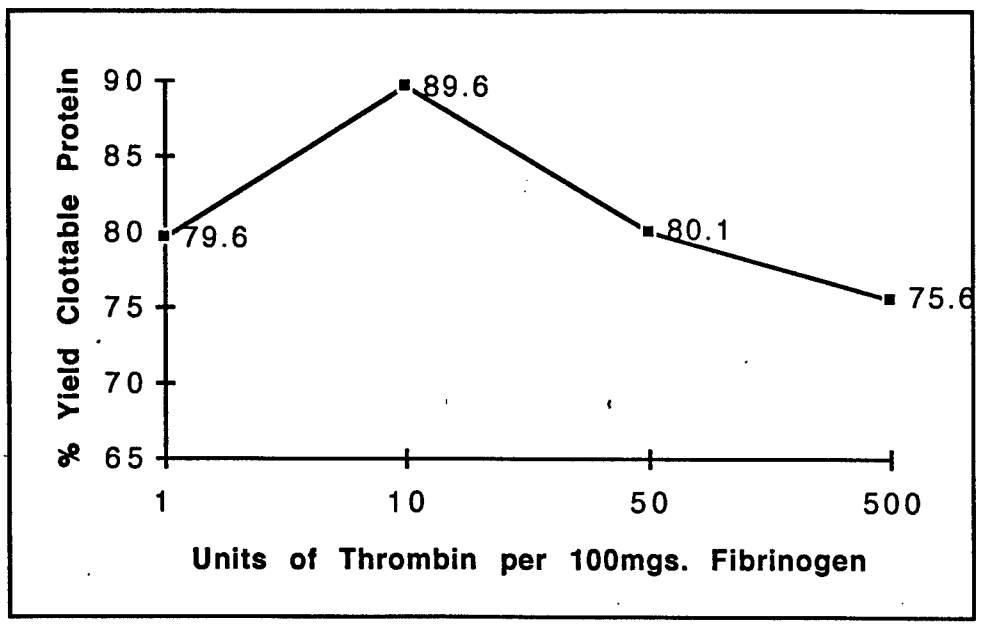


Figure 2. Fibrin yield versus thrombin concentration

Process Development of the Fibrin Formulation

To begin, each of the constituents were individually produced and analyzed with HPLC methods to acquire characteristic "footprints" of the components. These were used later to identify the constituents when placed together in solution.

The HPLC analyses were performed with the use of a Waters HPLC model 510 isocratic pump with a Shodex protein KW-803 column (8 mm x 300 mm). The packed columns of the Shodex protein KW-800 series are designed to be used in high performance chromatography for separating proteins, enzymes and polysaccharides. The mobile phase was 0.9% NaCl

and a flow rate of 5 ml/min was generated with a Waters Solvent Delivery Module (Model 590). Fifty microliter (50ml) injections of the sample were introduced through a Waters U6K injector.

The amount of sample present was quantified by means of a Waters 450 Tunable UV Absorbance Detector, set at 250 nm and 1.0 AUFs, connected to a Waters Data Module (Model 730). The Data Module is a microprocessor based printer/plotter/integrator designed to provide quantitative information for HPLC applications.

The mobile phase was characterized by injecting a 50 ml sample of the 0.9% NaCl solution. After acquiring the characteristic pattern of the saline, the testing of other agents could proceed.

Solutions of the above mentioned constituents were created for characterization as follows. Thrombin (Thrombostat, 20,000 Units NIH/20 ml isotonic saline, Parke-Davis) was compounded to provide a concentration of 1 mg of thrombin per 1 ml of 0.9% sodium chloride. Fibrinogen (fibrinogen fraction 1 Type IV: from bovine plasma, Sigma Chemical Company, St. Louis MO.) was reconstituted to provide a concentration of 1 mg of fibrinogen per 1 ml of 0.9% sodium chloride. Aprotinin is available in solution (Trasylol®; Miles Inc., West Haven, CT) and was used directly.

Fibrin monomer was obtained from the products of the reaction between fibrinogen and thrombin. As in the final stages of the coagulation cascade, the plasma protein, fibrinogen, is cleaved by the protein enzyme, thrombin. Fibrinogen is a symmetrical molecule containing three pairs of non-identical peptide chains, the overall structure being (Aa, Bb, γ)2. The chains are held together by disulfide bridges. The proteolytic attack of thrombin on fibrinogen is highly specific and limited. Thrombin cleaves an arginyl-glycine bond in each of the a and b chains, releasing two A peptides and two B peptides. The remaining portion of the molecule is termed fibrin monomer. The fibrin monomer has the automatic capability of polymerizing with other fibrin monomer molecules, forming long fibrin threads within seconds. Therefore, such a reaction will produce a peptide solution with a fibrin coagulate.

To accomplish this, the fibrinogen and thrombin solutions were combined in a 1:1 ratio and allowed to coagulate. The resulting formulation was then subjected to centrifugation and the resultant supernatant decanted off. The supernatant was reserved to obtain a characteristic trace of the cleaved peptides.

The remaining fibrin coagulate can be decomposed back into fibrin monomer molecules with the use of urea, high salt concentrations, or pH extremes. Fibrin coagulate can be decomposed back into fibrin monomer

molecules with the use of urea, high salt concentrations, or pH extremes. This is because the early stages of polymerization are due to loose hydrogen and hydrophobic bonding which can be broken apart with ease under these conditions. In attempting to isolate the fibrin monomer, the resultant coagulate from the above example was subjected to a solution of 8 M urea.

It has been shown that an 8 M solution of urea and a 0.1 M solution of acetic acid will dissolve the coagulated fibrin and thrombin, thus releasing fibrin monomer. However, subsequent lyophilization of the solution resulted in inseparable fibrin monomer and urea crystals. Consequently, the fibrin monomer was dissolved in a solution of ammonium hydroxide (Na4OH) (Aldrich Chemical Co., Inc., Milwaukee, WI). Lyophilization of this solution resulted in the evaporation of the ammonium hydroxide, leaving the fibrin monomer behind as a white powder. The monomer was reconstituted in saline solution for characterization with HPLC methods.

These solutions of the hemostatic agent constituents were analyzed on the HPLC to ascertain the characteristic footprint of each. Fifty microliter (50 µl) samples of each solution were injected for chromatographic analysis. Blanks of the reconstituting solutions were also run to verify their presence. This same procedure was repeated three times to assure reliability and accuracy. Consequently, the following constituents were identified with HPLC methods: thrombin, fibrinogen, aprotinin, saline, fibrinopeptides A and B, and fibrin monomer. These will be used later to confirm the composition of bathes of the agent prior to lyophilization.

The method for we have determined for preparing fibrin monomer is as follows:

Lyophillized fibrinogen purchased from Sigma chemical company was combined with thrombin (Thrombostat) supplied by Parke Davis. The mixing ratios are:

Fibrinogen	110 mg
water	1 mls
reconstituted Thrombostat	10 NIH units
Ammonium hydrox.(10%)	10 mls

Final Fibrin Solution Production/Manufacturing Process

110 mg of fibrinogen is first dissolved at room temperature in distilled water and sonicated briefly, if necessary. Similarly, Thrombostat is rehydrated as per Park Davis instructions. After dissolution of the two

solutions, 10 units of Thrombostat solution is injected into the fibrinogen solution, the two react almost instantly to form a soft gel.

To this gel is added 10 mls of a 10% ammonium hydroxide solution, this mixture is sonicated or shook until the gel dissolves. The dissolved fibrin monomer can then be lyophilized to dryness or it can be immediately dispersed into a polymer solution. If the fibrin monomer solution is not used immediately our work indicates stability for up to 2 weeks if properly refrigerated.

Sterilization of Final Fibrin Solution.

The need is now defined as to that of finding a suitable method of sterilization of the final lyophilized powder form of the fibrin monomer. A solution of the lyophilized fibrin monomer was prepared according to protocol. Two batches of fibrin monomer were made using 10 units of thrombin to 100 mgs of fibrinogen for gamma irradiation experiments. 85 milligrams of product were recovered after dissolving in ammonia and filtering with a 5 um filter. The resultant clot was then lyophilized overnight and washed with distilled water and filtered through 10 um filter. The remaining slurry was then dried in a vacuum, weighed, and separated into two parts consisting of 40 mg and 45 mg respectively.

The powder was then divided up with a portion of the powder placed in quarantine for later comparative testing with the sterilized amounts. One portion of the lyophilized powder was placed in a test tube and irradiated with 2.5-5 megarads of gamma radiation. The powder was returned to the laboratory and tested. HPLC results of samples sterilized by gamma irradiation were compared to control samples from the same lot. Results showed a slight shift in the elution time in the sterilized samples but the peak shapes were similar for both the control sample and the irradiated sample, as evidenced in the included chromatographs.

HPLC results using the same methodology obtained for the fibrin monomer samples sterilized by gamma irradiation were compared to results for control samples from the same lot . Results showed a slight shift in the elution time in the sterilized samples but the peak shapes were similar for both the control sample and the irradiated sample.

First attempts using a single reversed phase uBodapak C-18 column (3.9 X 150 mm) indicated that both the control and the irradiated sample exhibited more than one peak.Detection was performed at a wave length of 206 nm using 2.0 ABS units and a column loading of 25ug of fibrin monomer.

Attempts at improving peak resolution by changes in the mobile phase proved fruitless, but better resolution was obtained using two

columns in train utilizing the above operating parameters. Figure 3 shows typical chromatograms of both the control and the gamma irradiated samples. The control shows two differentiated peaks at retension times of 2.23 and 2.45 as well as the appearance of an emerging shoulder. The irradiated sample shows three separate peaks at retention times of 2.16, 2.56 and 2.91.

This data appears to indicate that gamma irradiation alters the chemical structure of the fibrin monomer, but whether such changes may affect the properties of the fibrin monomer is not decernable by this analysis. No attempts were made to identify the constituents of the irradiated sample or compare them with reported fibrinolysis products, as it seems unlikey that the denatured protein would retain its normal activity and function in the tissue adhesive. Batch sterilization using this method is thus not usable. We, therefore, will rely on sterilization by filtration of the solution before lyophilization.

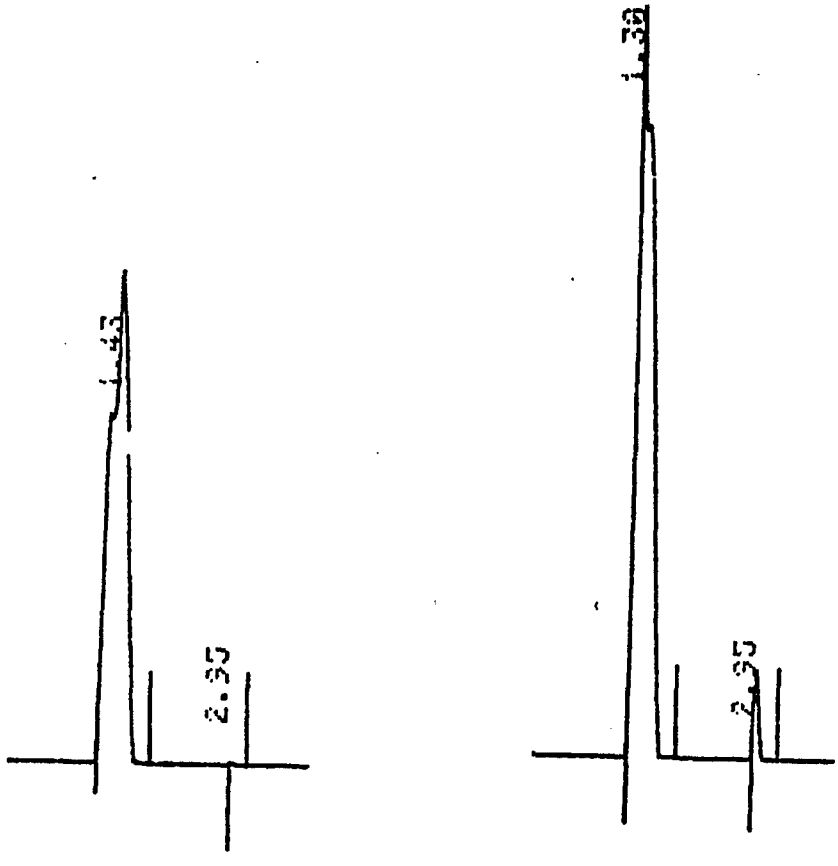


Figure 3. Chromatographs of non-irradiated fibrin monomer on the left and gamma sterilized fibrin monomer on the right. This method of sterilization appears to denature the protein.

TaskII. Chronic Tissue Healing Studies

Chronic tissue healing studies are currently being conducted at the Cleveland Clinic, Cleveland Ohio, under the direction of Hiro Harasaki M.D., Ph.D. The efficacy of the fibrin tissue adhesive is currently being tested and compared with Tisseel® in a rabbit splenic injury model.

The facilities at the Cleveland Clinic Foundation Department of Bioengineering, the site of the current animal studies, include a 15,000 sq. ft. laboratory with two operating suites and animal boarding space on site. This facility conforms to all Federal and local regulations concerning the care and use of laboratory animals.

The tissue adhesion produced by the fibrin tissue formulation is directly measured by measuring the tensile strength of surgical wounds created in the rectus femoris muscle. The methodology employed is similar to that which has been described in the literature to measure the adhesion of skin transplanted using a fibrin adhesive.

Under ketamine (35 mg/kg, IM) and xylazine (5 mg/kg, IM) anesthesia, the left flank is shaved and the skin is prepared using Betadine. The abdomen is entered through an incision placed along the left costal margin. The phrenicosplenic and splenocolic ligaments are dissected, if required, to mobilize the spleen into the operative field. Using a specially designed guiding device, four parallel scalpel incisions are made on the anterolateral surface of the spleen.

The incisions are of the same length and depth, involving both capsule and parenchyma. This sharp incision wound model has been chosen because of the reproducibility of the extent of the wounds created and the simplicity of the procedure. Although the spleen of the rodents may contract upon various physiological conditions and trauma, and the mode of bleeding and tissue healing may not be directly comparable with those seen in humans, the comparison of various formulations of the fibrin glue can be done in a sufficient manner with this animal model.

The first wound is treated with Tisseel®, and the amount of the fibrin glue needed and the time lapsed for complete hemostasis are recorded. The second wound is treated with formulation #1 in the same manner. The third and fourth wound is treated by the formulation #2 and #3 respectively. The amount of preparations used and the time for the complete hemostasis are recorded as well. The abdominal wall is then closed in layers using conventional sutures.

All the surgical procedures are performed under the sterile conditions. The post operative care includes a watchful observation for the

signs for internal bleeding for the immediate postoperative period, prophylactic antibiotics, a mixture of benzathine-procaine penicillin (40,000 u/kg, IM) for two days and oral food and water intake, ad libitum, after recovery from anesthesia.

Usage of anti-inflammatory drugs, which modify the inflammatory response and tissue healing, will be avoided. Postoperative pain will be treated with intramuscular meperidine, 5mg/kg, p.r.n..

A total of 36 rabbits have been divided into 3 groups;

Group 1. The specific aim of this group is to observe mainly the hemostatic effects of fibrin glue preparations. The animals will be sacrificed after 48 hours with the overdosage of intravenous pentobarbital injection. The spleen will be carefully examined for any persistent bleeding and the sizes of each wound and accompanied hematoma. The spleen will then be removed and weighed. A cross sectional tissue specimen will be obtained from the center of each wound for histological and morphometrical evaluation of the size of the wound, degree of internal bleeding and the extent of inflammatory responses. Twelve animals will be used.

Group 2. The specific aim of this group is to evaluate the rate of tissue healing and the inflammatory response to each formulation after one week of injury. After inducing the splenic injury, twelve animals will be kept for one week and the macroscopic and microscopic evaluations will be performed in the same manner as in the Group 1.

Group 3. The specific aim of this group of experiment is to observe the healing process at the one month post-injury period. Twelve animals will be used.

Tissue specimens are obtained from each animal and each injury site as mentioned above. The specimens are then fixed in HistoChoice, which is suitable for immunohistochemistry, when indicated, while preserving the tissue for reasonable conventional morphological studies. The plastic embedded specimens are sectioned, and stained with hematoxylin-eosin and with Masson's trichrome. The area of granulation and fibrous scar from each animal is quantified using a BioQuant Morphometric System (R&M Biometrics Inc., McHenry, IL).

These studies were begun late in the most recent quarter. Results will become available by the next quarterly report and at regular intervals thereafter.

Task III. Delivery System Development and Testing

Task III included the formulation development for the hydrophilic foam, designed to be the scaffold for the fibrin monomer based tissue adhesive. The development, formulation, and subsequent testing of this delivery system, has provided for the completion of a delivery system which encompasses both the foam sponge and fibrin tissue adhesive.

The objective of this task was to integrate the new hemostatic agent into a complete, pre-packaged wound dressing system which will readily allow it to be used under battlefield conditions. This system includes two types of delivery modes to be used separately or in combination depending on the wound characteristics. For deep tissue injury, an elastomeric sponge loaded with the hemostatic agent has been designed to control bleeding, for superficial injury, an elastic, semi-occlusive dressing incorporating the hemostatic agent in a release layer is designed to control bleeding while covering and protecting the site of injury. Together, these dressings will provide superior wound stabilization and treatment.

Traditional dressings provide little protection from infection, in fact they often provide a supportive bacterial environment. By being absorbent, these dressings often dehydrate the wound. The exudate material is then absorbed into the dressing. Since these materials are not impervious to microorganisms, the saturated dressing may provide an excellent substrate supporting microbial growth. Consequently, infection is aided rather than prevented.

This situation may be overcome with the incorporation of antibiotics in the dressing to provide aseptic conditions at the wound surface. Traditional wound dressings have generally consisted of either sterile, absorbent, cloth pressure bandages or flat strips of elasticized, adhesive film. These materials have minimal beneficial characteristics and primarily function as simple coverings to protect wounds.

In both deep and superficial wounds, these dressings may provide a mild measure of hemorrhage control by the application of pressure. This is a physiological phenomenon that has little to do with the material bandage used. In this case, clotting is slow and uninterrupted pressure must be maintained for a long period of time. In the intense atmosphere of a battlefield setting, this may not be feasible since sustained periods of time and restricted body movement are incongruous with combat.

Polymer Foam Testing and Development

The third quarter report on this project provided two separate methods for the preparation of polyurethane foams suitable for use in this application. The first employed the single shot method where, as one would expect, all the reagents were mixed together and cast into a mold. The second method required the preparation of a prepolymer followed by further polymerization which was controlled by the addition of a chain extender.

Further work was performed in this period which helped to further define the open cell foam as a method of delivery for the tissue adhesive/hemostatic agent. The results of these tests indicated that the single shot method would provide the best vehicle from which to launch the production of the foam carrier. In order to reduce the chance of batch to batch inconsistencies it was decided to prepare one large block of foam prepared from the same lots of raw materials. Utilizing the single shot method, a foam sample large enough to provide sufficient samples for toxicity testing was made. This operation required the preparation of sheets totaling 3000 cm^2 in area.

The following reactants mixed at their stated weight ratios. First 580 gms of the polyol Pluracol L64 was added to a polypropylene cup having sufficient capacity to hold the liquid foam reactants. To this was added 12 gms water and 2 gms of catalyst T12. These three reactants were mixed with a mechanical stirrer and set aside for later use. In a separate polypropylene container 248 gms of Mondur CD was added. Both containers were allowed to equilibrate to ambient temperature 25^0C.

The container containing the polyol was replaced under the mechanical stirrer and the Mondur CD was added under rapid agitation. The mixture had a 45 to 55 second cream time. The stirrer was removed and the rising liquid was cast into a polyethylene lined rectangular mold, pre-heated to 37^0C. The foam was allowed to cure at this temperature for 3 hours.

The following day the foamed bun, occupying approximately one cubic foot, was removed in preparation for physical testing and the cutting of toxicity samples. The foam bun was cut into twenty sheets approximating 7 cm. X 22 cm X 0.5 cm and the remainder set aside. These sheets were dialyzed in buffered saline for 48 hours, the solution changed at the 24 hour mark. The dialyzed samples were patted dry and placed in a 50^0C oven for one hour to dry. The samples were then sterilized and packaged for shipment.

Task IV. Controlled Release of Antibiotics

Although hemostatic agents are designed for immediate activation, pharmacologic agents may be useful both immediately, to control initial infection, and over time, to prevent further infection. Therefore, antimicrobials may be present in both non-encapsulated and/or encapsulated forms for immediate release and sustained release, respectively.

Sustained or controlled release of drugs, or any additive, has been investigated for many years. The most mechanically simple and smallest release devices are those consisting of polymer matrices. These matrices may be in the form of tiny spheres. Here the release rates are determined by the diffusion rates of the additive in the polymer or, as in a bioerodable system, by the degradation rate of the polymer. Many times the combinations of both occur simultaneously. So once the additive has been selected, the polymer choice is of utmost importance. These polymers must be non-toxic, be able to be processed into usable matrices by familiar methods, and must not alter the additive function. Three common polymers used for controlled release systems are poly(DL-lactide co-glycolide) (PLGA), polymethylmethacrylate and polycyanoacrylates. All of these however, are to brittle, degrade slowly, or have effective release periods much different than those needed for a short term dressing (one week maximum).

It appears reasonable that controlled release of drugs, or other additives over short periods may be attainable through the encapsulation of drugs into soluble polymer capsules. These microspheres can be dispersed into a flexible polymer film suitable for a dressing, and should have a well defined elution rate over a maximum 4 day period.

Antibiotic Selection

Meaningful statistical data from soldiers in the field with combat casualty infections are rare. This is understandable, since in wartime there is little time to conduct statistically designed bacteriologic surveys. Wound infections are customarily treated without culturing, and only those infections that prove resistant to conventional therapy are ultimately cultured.

Infections in the field will usually involve a mixed flora. Bacterial contamination in field wounds typically involves normal skin flora, dirty clothing and sweat contaminants, fecal soiling contaminants, various soil organisms depending on geography and region, other resident environmental flora, and organisms found in standing water (many gram-

negative organisms; i.e., Pseudomonas, Serratia, enterobacteria, Herellea, Flavobacterium).

Antibiotic-loaded field wound dressings must be capable of controlling bacterial infection in contaminated wounds, or be able to control established infections such as those encountered in dirty and infected wounds.

Thus, the selection of topical antimicrobials for use as battlefield wound dressing is quite different from the selection process involved in conventional systemic therapy. The ideal topical antimicrobial agent should be poorly absorbed through skin, for maximum kill potential at the applied site, and be bacteriostatic at low local concentrations.

No one agent has activity against both bacteria and fungi. However, there appears to be several suitable combinations which can bridge the gap. One such pair is chlorhexidine gluconate (CHX) and nystatin. Indeed this combination has been shown to be effective against S. aureus, S. pyogenes, P. aeruginosa, C. Albicans and Trichophyton species.

The primary use and design of the field dressing is to be for emergency conditions where immediate stabilization of the individual awaiting evacuation is requisite. Therefore, the initial objective of the dressing is to control bleeding, then to prevent infection by controlling local bacteria with the use of antimicrobials. The proliferation of fungi occurs in the absence of local bacteria. However, it is unlikely that this will occur in the time span that the dressing is to be worn. Therefore, a dressing containing a single antimicrobial, such as CHX should provide ample protection until the individual is given professional medical aid.

The second type of field dressing currently under development is a silicone film supporting the hemostatic agent and supporting or containing an antimicrobial for the treatment of superficial wounds. This film is intended to be used for topical treatment of superficial wounds to provide temporary hemostasis, infection control and physical protection until further treatment may be undertaken.

The topical dressing consists of three layers: 1) a top layer consisting of a self adhering, unsupported, polyurethane bandage acting as a protective barrier, 2) a silicone island, possibly loaded with dispersed and/or encapsulated CHX. 3) a removable blood soluble adhesive layer on the surface of the island acting as a binder for the lyophilized fibrin monomer/thrombin mixture developed in Phase I and possibly encapsulated CHX. The adhesive binder will release the coagulant with initial blood contact.

The development of the polyurethane barrier film is not included in this proposal as many commercial films already exist which are suitable. Attachment of the medicated films to commercially available semi-

occlusive urethane films should lead to a more rapid product development. One of these, ARCARE 7560, is a transparent polyurethane which will be used as the barrier coat. ARCARE 7560 is a semi-permeable membrane which we can modify by coating it with a pressure sensitive adhesive. The adhesive coated polyurethane film can then be qualified further by extensive adhesive testing before final integration into the dressing.

The island matrix films will consist of a new family of biomedical-grade oligomers. These oligomers are ideal for the design of a military field wound dressing for the following reasons:

- Oligomers are synthesized from non-exotic, off-the-shelf chemicals; thus, the dressing will be inexpensive.
- All the necessary manufacturing technology is present at Whalen Biomedical; therefore production lead time will be minimal.
- The field wound dressing can be applied by non-medical personnel.
- The dressing is highly compliant for physical comfort.
- The dressing will help achieve hemostasis and infection control and will promote normal wound healing.

Such films have oxygen, carbon dioxide and water vapor permeabilities resembling those of intact skin. In addition, these liquid oligomers may be precompounded with coagulants and antibiotics, thus behaving as extended-action, controlled-release formulations.

An important technical aspect of our development of non-toxic, tissue-compatible oligomers is that they cure at room temperature. Solidification at room temperature is a vital consideration because most drugs, such as antibiotics, are rapidly inactivated upon mild heating. To insure full pharmacological activity, the antibiotic is not subjected to heat.

This requirement was met by incorporating the antibiotic into the liquid matrix of the uncured oligomer which will solidify upon mere exposure to atmospheric humidity at room temperature. Once cured, the oligomer becomes a controlled release monolith, capable of dispensing the antibiotic at a continuous and predictable rate. Thus, utilization of such moisture curing oligomers permits the production of ingenious drug-dispensing wound dressings.

In our technology we utilize a physical mixture of silicone-terminated oligomer and a low molecular weight silicone fluid. The silicone fluid acts as a fugitive excipient, which exudes to the surface, reducing the tendency of clots from adhering to the dressing. This facilitates removal of the dressing without causing additional bleeding.

Syntheses Of Silicone Terminated Oligomer

We can prepare silicone terminated polyols by curing prepared polyoxypropylene films via a silicone moisture reaction. Such copolymers are prepared from the condensation reaction of the terminal methoxy and hydroxyl groups on each starting monomer respectively. The terminal methyldimethoxysilane groups can undergo further reaction with water under catalytic conditions to crosslink the system. This concept was based on earlier work that has documented the elution of antibiotics from a polyoxypropylene polyether matrix containing poloxamer182 reported for a 20% loaded film.

The molar ratio of silicone to polyoxypropylene was held at 2:1 as shown below.

2(3HCO)3HCSi-(Polyoxypropylene Mw)-SiCH3(OCH3)2

The weight ratio between the reactants was controlled by varying the molecular weight (Mw) of polyoxypropylene from 5000 to 8000 Daltons. The molecular weight of polyoxypropylene is determined upon reaction of trimethoxymethylsilane with a polypropyleneglycol (PPG). By varying the molecular weight of PPG, a variety of PPG oligomers may be synthesized. Such variation may also be used to maximize those properties most desirable in a field wound dressing, such as tensile strength and hardness.

This was accomplished during the last few years, using well-known principles of polymer chemistry for guidance in such variations. These principles state that as the molecular weight of the PPG increases, the tensile strength and hardness concomitantly decrease. Thus, the properties of the finished, cured film may be tailored by varying the molecular weight.

As the Mw of polyoxypropylene increased from 5000 to 8000, the weight ratio of polyoxypropylene increases from 94 to 98% of the weight of the copolymer. The lower number corresponds to the molecular weight of an earlier oligomer which has shown promising results in previous efforts; the higher value of Mw was chosen as a limiting value because the viscosity of the polyoxypropylene oligomer increases greatly and approaches being a solid which could give problems in the additive(s) compounding step.

Formulation Test Matrix

Three initial copolymer formulations were prepared using polyoxypropylene with a molecular weight of 5000, 8000, and 9000.

Enough material was prepared for each formulation to allow characterization of the polymer, preparation of drug loaded film, and to maintain an inventory.

The three starting neat formulations were the liquid oligomers, 5000, 8000, and 9000 based, containing 1% by weight CHX. These were cured and tested for drug elution rates. The better of the three was chosen and a larger batch prepared and kept as the control for future development.

A master batch of CHX powder was mechanically dispersed into a USP excipient, PEG 8000, to a solids concentration of 50% by weight. The CHX concentrate was prepared by slowly adding the powder into the excipient and grinding into a uniform dispersion by means of a mortar and pestle. The resultant concentrate will be examined for the quality of dispersion by photomicrographs and this drug concentrate will be used in all ensuing matrix formulations.

Once drug elution rate and microbiological activity has been identified, the best CHX concentration will be determined. We will prepare additional matrix formulations increasing the drug/poloxamer concentration until a maximum level of gentamycin is eluted, the maximum concentration for gentamycin in this case would be 25% by weight.

The final mixture will be applied in the form of a 250μm-thick membrane onto a release liner coated with an acrylic adhesive. The oligomer layer will be cured in our autoclave held at 25°C, by exposure to 50% relative humidity. The cured film will be removed the following day and covered with a continuous polyethylene sheet to protect the cured membrane from airborne contaminants. Island patches will then be dye cut into one inch squares and centered onto the adhesive surface of the polyurethane films.

Test Protocol for Elution Studies

Polymer films were prepared and samples were die cut using a 1.125 diameter steel die. These were weighed on a Mettler balance and the weights recorded. Theoretical drug loadings were calculated using the weight of the film samples and the assigned drug loadings. Two samples of each of the films were tested by placing the die cut sample into a closed flask containg 20 ml of distilled water. These flasks were placed in a constant temperature bath set at 38C under constant agitation. Samples for HPLC analysis were withdrawn at intervals of 1, 2, 4, 8, 24, 48, 72, and 96 hours. Results, for each time period, were reported in percent of theoretical loadings of drug eluted per square centimeter of exposed film per ml of extraction media.

Preparation of CHX Concentrate for use in Film Elution Studies

Chlorhexidine gluconate was encapsulated into a drug excipient in order to increase both the control of drug release as well as the rate of release. PEG 8000 was chosen for the drug excipient at a ratio of 3:1 PEG/CHX respectively.

The following methodology was used to prepare enough encapsulated CHX to run the series of elution tests:

PEG 8000 was placed into a freeze dryer jar dissolved in a stock of solution of CHX 20% in water. To this was added an equal weight of distilled water to yield a total solids of 5%. The 5% solution was then frozen. The frozen sample was placed into the freeze dryer and vacuum dried overnight. The following day the sample was removed from the jar and the sides of the jar scrapped clean. The material was white with no noticeable inclusions or color and had a fluffy texture. The white powder broke up easily and initial downsizing was attempted using a mortar and pestle. Later it was determined that an electric grinder did an adequate job and the resultant powder was sieved through a series of 180, 150 and 90 microns. The material that did not pass through the 90 micron sieve was placed into the grinder and reprocessed. When enough powder was collected it was placed in a vile for later use in film preparation.

Summary of CHX Elution Studies

The final polymer choice and antimicrobial loading was selected as the 8000 Maw formulation containing 2% loading which appears to be a good balance between potential efficacy and product cost. Because the final use of this dressing is intended for personnel who may be exposed to environments with extremely high bioburden it is felt that a rapid elution of the CHX or antimicrobial could be advantages. It is this rapid dumping of the 8000 Mw film as well as its ability to continue to elute antimicrobials over the 96 hour test period which make it a good choice for this application. Concentrations of 2 and 3% CHX in a test formulation showed parallel release rates with the absolute values proportional to concentration. However, there is a 33% saving in the cost of the antimicrobial using the 2% loading which in the future may be requisite.

Results of Elution Tests Using CHX as the Antimicrobial

A: Results of Elution Tests Employing Different Mw Polymers

Fours polymers were selected to prepare films for the inclusion of CHX to study the elution rate over time. Three of these were liquid oligomers prepared from polyoxapropylene backbones which were subsequently cured into films via the hydrolysis of terminal siloxane groups. These three varied in molecular weight from a low of 5000 to a high of 9000 Daltons. The fourth choice was a commercially available two component low temperature curable silicone elastomer (Applied Silicone 40071). The following graph shows that the 9000 Mw and the 8000 Mw films were able to elute much greater percentages of CHX as compared to the 5000 Mw and silicone films. Also the 8000 Mw film dumped the CHX much faster initially than the 9000 Mw film as well ultimately eluting a higher final percent of drug (90% vs 70%).

These results are shown graphically in Figure 4

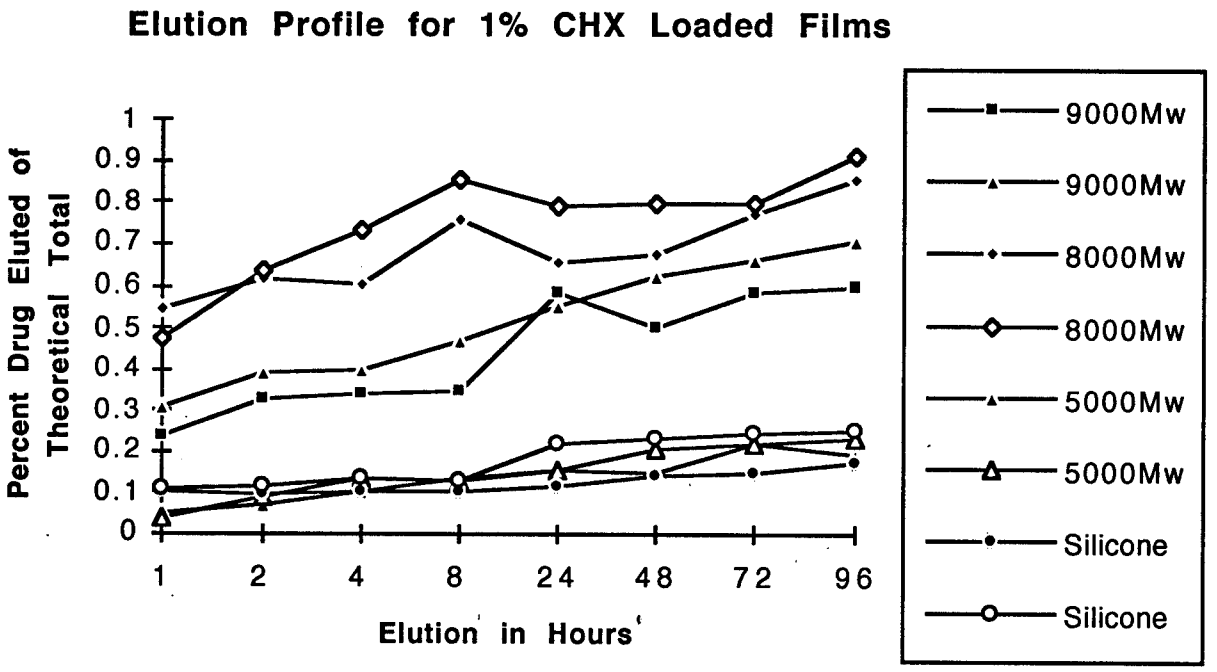


Figure 4. Drug elution rates for the various polymers examined.

B: Results of Elution Tests for Varying Drug Concentrations

Trials were performed to determine the effects of drug concentration on the elution rate from a randomly chosen polymer formulation. These were performed in parallel with the above elution tests so the best formulation was unknown at the start. Figure 5 below shows: the drug elution rate increased dramatically going from 1% to 2 or 3% drug concentrations. Formulations containing 2 and 3 percent antimicrobial

are best for rapid early release. These formulations showed parallel release rates between the two with the absolute values proportional to concentration. i.e. 2.7 release for the 3% and 1.45 release for the 2%.

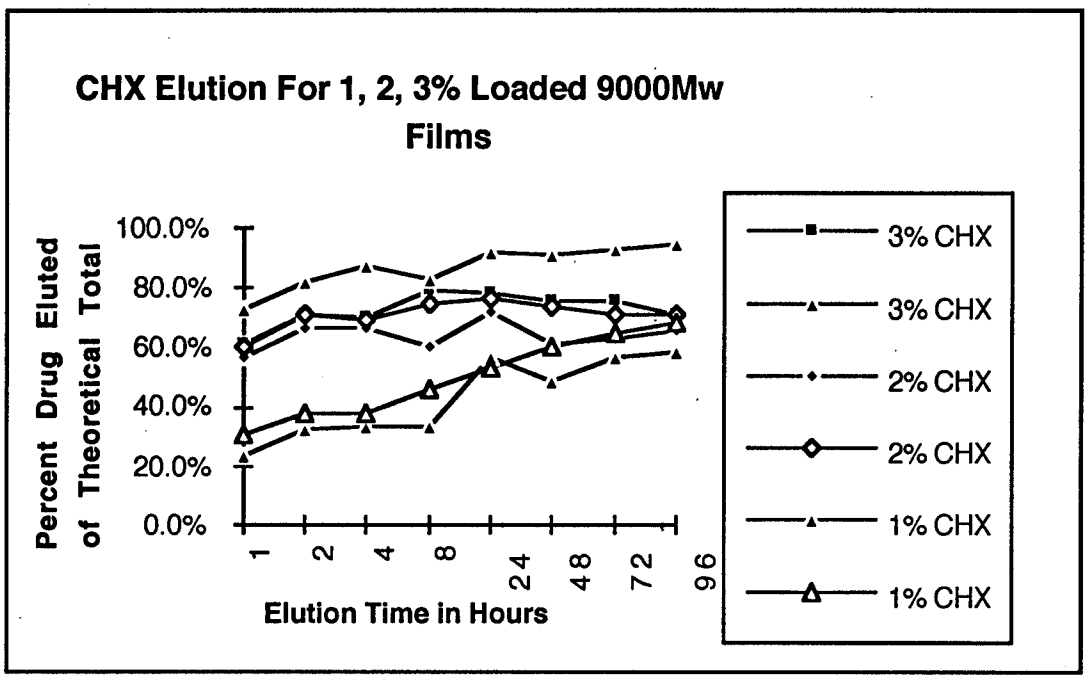


Figure 5. The effect of drug concentration on elution time.

Physical/ Chemical Properties of the Copolymer

The final formulation for the polymer will be analyzed in both the liquid prepolymer state and the cured state to quantify the basic properties of the polymer. The bulk properties of any polymer are related to its molecular weight (Mw). In the uncured state the Brookfield viscosity is a rapid measure of the Mw and can be correlated to quantitative Gel Permeation Chromatography (GPC) results. The Mw of the polymer may be measured in the liquid prepolymer state with the use of GPC. This method will require the use of a column train consisting of four gel columns with 5 mm particles 10^4 A, 10^3 A, 500 A and a 100 A (7.8 mm ID x 30 cm). Toluene will serve as the mobile phase with a flow rate of 1 ml/min, generated by a Waters 590 pump. A Refractive Index detector will also be necessary.

First, a series of polystyrene standards of known molecular weights will be analyzed to generate a calibration curve. Then, the test samples will be analyzed to determine their corresponding molecular weights. Output data will include peak results representing the mixture of molecules in the uncured polymer having a range of molecular weights. Several molecular

weight averages will be calculated based on the integrated peak. Different mathematical manipulations of the data yield various molecular weight averages, including the number average molecular weight (Mn), weight average molecular weight (Mn), and the polydispersity (Mw/Mn). Polydispersity defines the width of homogeneity of the polymer with unity being the theoretical value. However, as a polymer degrades this value increases from its characteristic value, its distribution becomes broader and its physical properties decrease. GPC analysis will be used to characterize each formulation prior to the curing of each into their respective insoluble films.

Once the polymer has cured, the Mw is difficult to obtain. However, in this state, physical properties such as tensile strength and percent elongation may be measured to characterize the material and determine the extent of the cure. For these tests, polymer samples will be prepared as 0.5 mm thick films which have been cast on release paper. The tensile strength and percent elongation for each formulation will be determined employing ASTM D882-64T procedures.

The chemical structures of the polymers will be identified by Infrared Spectroscopy. A fingerprint spectrum of the individual polymers before and after curing will be prepared for use as reference spectrums and any unusual features will be noted.

In addition, it will be necessary to perform the above tests on the base polymer without the antibiotic present. This will establish a baseline value for each antibiotic/polymer formulation and the effect of antibiotic inclusion. Initial values for the tensile strength and percent elongation for the 8000 Mw were determined to be 250 psi and 550%, respectively. The viscosity range and tensile strength will be determine for each formulation without the antibiotic present in order to create a baseline. The effect of including the antibiotic, if any, should be discernible by comparison to the base values.

Moisture vapor transmission rates will be determined for both the cured polymer films and the polymer film/polyurethane composite using ASTM E 96-66 testing protocol.

Adhesion to Skin

Skin adhesion is a fundamental property required to hold any device in place. However, adhesive properties should be such that the dressing can be removed after the required residence time in an unremarkable manner. Skin adhesion should be balanced between: (a) the adhesion level required for secure holding regardless of patient movement, perspiration level or bathing, and (b) ease of removal when dosage is complete.

The most desirable adhesive system will also show uniform adhesion to skin over time with only moderate adhesion buildup or loss. Also, the range of values observed should be statistically reproducible and as small as possible.

Adhesion level to the patient dosage site with a wound dressing should only be enough to effectively keep the device in place for the necessary dosage period. Higher levels of skin adhesion should be avoided where possible, since high skin adhesion levels increase the incidence of excoriation during removal. Higher than necessary levels of skin adhesion also increases the probability of skin sensitization and irritation with repeated use on the same site. In our medicated wound dressing, we have chosen an acrylic-based, pressure-sensitive adhesive that builds adhesive to the skin site rapidly, plateaus, and thereafter maintains uniform adhesion for up to seven days. Upon removal, we have observed a minimum to adhesive residue left on the skin site, and removal has been unremarkable.

Cohesive Strength

This is the ability of the adhesive to stay together and to stay in place under load, i.e. resist shear. Good cohesive strength is also vital for clean removal from the skin with minimum residue. It is a manifestation of the visco-elastic properties of a particular system.

Cohesive strength is a function of the molecular weight and molecular weight distribution. Addition of relatively low molecular weight tackifying agents to compounded adhesives affects the molecular weight distribution. Adhesive processing during coating can also directly influence final molecular weight distribution.

Positive tests for good cohesive strength in vivo are the unit staying in place on the patient (not sliding) and unremarkable removal with no visible adhesive residue left on the skin.

In our wound dressing, we selected a pressure-sensitive adhesive which displayed sufficient cohesive (or internal) strength to remain in place, yet it peeled from the skin cleanly. Also, cohesive strength was not adversely affected by either ethylene oxide or gamma radiation sterilization, and it was unaffected by temperatures between 95°F and -20°F.

Anchorage of Adhesive

The pressure-sensitive adhesive, which is designed to hold a medicated wound dressing to a soldier's skin, must on the obverse side stay

adhered to the dressing. Keeping the adhesive firmly attached to the dressing is referred to as adhesive mass anchorage.

Adhesive mass anchorage is most easily tested in a direct manner. The tests are essentially qualitative; either it is satisfactory or it is not. An effective test that can be done without instrumentation is to simply fold the adhesive film composite pressure-sensitive side upon itself and press together to ensure good contact. Then peel one end back on itself creating a 180 degree peel test. Outcomes other than clean separation constitute failure.

Adhesive Qualification

Most commercial polymer dressings are composites of thin urethane films coated on one side with a medical grade pressure-sensitive adhesive. The advantages of these composites are their light weight, conformability and high moisture vapor transmission rates(MTVR) while still remaining occlusive to water and bacteria. The choice of acrylic pressure sensitive adhesive over other polymers is based upon the fact that these adhesives are commercially available in medical grades and that these adhesives are hydrophobic and can maintain their adhesive strength after immersion in water.

Adhesive transfer tapes manufactured by Adhesive Research Inc., under the brand names ARCARE 7560 and 8311 have shown they may be likely candidates. These medical grade adhesives have typical MTVR's of 500 to 1000 g/m^2/day respectively, and been shown to retain high adhesive properties even after immersion in water for 72 hours. Twelve test samples were prepared by bonding the adhesive coated polyurethane membrane to test strips prepared from sheep's skin, using both ARCARE adhesive composites. Six samples were maintained at ambient conditions, the remaining six samples were immersed into water and conditioned for 72 hours. Peel tests will be performed using the test assembly shown in Figure 3 per ASTM D-930 procedure. Results of wet and dry samples were compared and the percent retention determined.

Results

The group using the ARCARE 7560 showed the highest peel strength for both dry and wet samples. This group did loose 50% of its dry peel strength after exposure but the immersed samples still exhibited high peel strengths of 27 g/cm. All samples were listed as adhesive failure to the leather substrate.

The Arcare 8311 showed an increase in the peel values for the wet samples compared to the dry ones. But, the absolute values compared to the 7560 80 to 50% lower. The Arcare 8311 also exhibited adhesive failure to the urethane film as opposed to adhesive failure to the leather substrate. For these reasons, the Arcare 7560 was chosen to prepare test samples for storage stability testing. This data is shown in Figure 6.

Arcare	g/cm	Average	Trial# 1	Trial# 2	Trial# 3	Trial# 4	Trial# 5	Trial# 6
7560	Mean	54.7	38.7	55.0	53.3	62.0	57.7	61.4
Dry	SD	7.7	11.6	8.1	7.1	4.6	11.0	3.8
	Max	62.4	48.6	61.4	59.1	66.8	73.8	64.5
	Min	36.2	33.8	30.4	32.1	47.5	29.1	44.2
Arcare								
7560	Mean	27.3	26.4	29.8	31.2	22.4	25.6	28.3
wet	SD	9.0	8.0	9.8	11.2	7.2	8.3	9.4
	Max	40.7	39.5	44.3	43.1	35.6	38.7	42.7
	Min	11.7	10.3	11.9	1.7	8.2	29.1	8.8
Arcare								
8311	Mean	10.1	8.4	7.0	11.3	11.2	11.7	11.1
Dry	SD	0.9	.2	.3	1.8	.5	1.1	1.2
	Max	11.6	9.0	7.4	16.2	11.9	12.6	12.3
	Min	7.2	8.0	6.3	8.2	9.5	5.0	6.5
Arcare								
8311	Mean	14.5	15.5	14.3	17.0	14.3	13.3	12.8
Wet	SD	1.2	.7	1.4	1.3	1.3	.9	1.6
	Max	16.3	16.3	15.9	18.3	18.3	14.5	14.2
	Min	9.6	11.9	8.0	11.3	11.3	8.4	6.7

Figure 6. Summary of Peel Tests for Dressing Substrate.

The second type of dressing is a silicone film supporting the hemostatic agent and containing an antibiotic for the treatment of superficial wounds. This film is intended to be used for topical treatment of superficial wounds to provide temporary hemostasis, infection control and physical protection until further treatment may be undertaken.

The topical dressing consists of three layers: (1) a top layer consisting of a self adhering, unsupported, polyurethane bandage acting as a protective barrier; (2) a silicone island, possibly loaded with dispersed and/or encapsulated gentamycin; and (3) a removable blood soluble adhesive layer on the surface of the island acting as a binder for the lyophilized fibrin monomer/thrombin mixture developed in Phase I and possibly encapsulated gentamycin. The adhesive binder will release the coagulant with initial blood contact.

Task V. Packaging, Shelf Life, and Toxicity Evaluations

In this reporting period, toxicity tests of the foam delivery system were completed. These tests were conducted in the facilities of NAmSA (Norwood, OH), a commercial toxicological testing laboratory using standardized protocols. Samples of the foam sterilized by steam autoclaving were prepared at WBI and supplied to NAmSA. The same lot of material was used for all of the tests which were conducted. Because of potential heat sensitivity of the material, we now employ ethylene oxide gas sterilization for this purpose.

Results of Toxicity Testing

Initial testing began with the in vitro hemolysis test. A 85.2 square centimeter portion of the test foam was placed in 28 ml of 0.9% sodium chloride solution and extracted at 70° C for 24 hours. The extract was then divided into individual tubes of 10 ml each and allowed to cool to room temperature. To duplicate aliquots of the extract and to a similarly treated set of positive and negative control tubes was added 0.2 ml of rabbit blood previously collected in a vacuum tube containing EDTA. The tubes were inverted gently to mix the contents, then placed in a constant temperature water bath at 37° C for 1 hour. The blood saline mixture, positive and negative controls were then centrifuged for 10 minutes at a speed of not less than 1000 x g.

The absorbance of each test article solution was determined spectrophotometrically at 545 nm. Similarly, absorbances were recorded for the positive control (10 ml water and .2 ml blood) and the negative control (10 ml 0.9% sodium chloride solution and .2 ml blood). Absorbance values for controls were used to calculate percent hemolysis for the test article.

Results showed 0.0% hemolysis for both test #1 and test #2, with a mean hemolysis of 0.0%. Under the conditions of this test, the test article would be considered nonhemolytic. Both the negative and positive control values were as expected. The test extract and control solutions were clear. All raw data pertaining to this study and a copy of the final report are retained in the NAmSA archive files. This study was conducted based on the procedure described in US Pharmacopeia 23, National Formulary 18, 1995. The study conformed to all applicable laws and regulations. Specific regulatory requirements included the current Good Laboratory Practices for Non Clinical Laboratory studies, FDA, 21 CFR, 58.

The intramuscular injection test for subchronicity has also been performed. This 5 day implantation test allowed for the evaluation of the polyurethane foam in regards to its ability to induce toxic affects in the muscle tissue of albino rabbits.

Using a sterile hypodermic needle and stylets four strips of the polyurethane foam were implanted into the paravertebral muscle on one side of the spine of each of two rabbits. In similar fashion two strips of a USP negative control Plastic RS were implanted in the opposite muscle of each animal.

The area of the tissue surrounding the center portion of each implant strip was examined macroscopically using a magnifying lens. Hemorrhaging, necrosis, discolorations and infections were scored using a scale from 0-3, with 0 being normal and 3 severe. The test would be considered negative if, in each rabbit, the difference between the average scores for each category of biological reaction for the test article and control article implant site does not exceed 1.0, or if the difference between the mean scores for all categories of biological reaction for each test article and the average score for all categories for all the control implant sites does not exceed 1.0, for not more than one of four test article strips.

The macroscopic reaction was not significant as compared to the USP negative control implant material. The average score for the foam implant was 0.0, indicating no difference between the foam test material and the implanted negative control. Under the conditions of this study, the macroscopic reaction was not significant as compared to the USP negative control implant material. The implant test article, foam, met the USP requirements.

A thirty day intramuscular injection test was also performed using the polyurethane foam. The study procedure is identical to that outlined above only instead of a 5 day evaluation time, a lengthened 30 day evaluation is scheduled based upon the average scores for the test articles and control article implantation site.

The USP intracutaneous toxicity study was also completed on the foam preparation. The test article foam was extracted in 0.9% sodium chloride USP solution, alcohol in saline, cottonseed oil, and polyethylene glycol. These extracts were evaluated for intracutaneous toxicity in accordance with the guidelines of the current USP.

A 0.2 ml dose of the appropriate test article extract was injected by the intracutaneous route into five separate sites on the right side of the back of each rabbit. Similarly, the corresponding blank vehicle was injected on the left side of the back of each rabbit. Two rabbits were used for each pair of extracts. Observations for erythema and edema were conducted at 24,

48, and 72 hours after injection. Reactions were scored on a 0 to 4 basis, 0 being no erythema or edema, and 4 being severe edema or erythema. Any reactions at the injection sites were also noted.

The cumulative average, erythema and edema score for each test extract and corresponding control blank was calculated. For each extract , a difference in average scores of 1.0 or less was considered to be acceptable. A difference of 0.6 to 1.0 indicated a slight but acceptable reaction. A difference of greater than 1.0 was considered to be unacceptable. Additionally the average score for each test extract and blank was calculated for each interval. Any adverse reaction noted in the test extract was compared to the corresponding blank.

The differences noted in the average scores was considered acceptable. The difference between test and control average scores did not exceed 1.0 at any observation interval. Under the conditions of this study, there was no evidence of significant irritation or toxicity from the extracts injected intracutaneously into rabbits. Each test article extract met the USP requirements.

Concluded also were the USP systemic toxicity studies. The test article, Foam, was again extracted in 0.9% Sodium Chloride USP solution, alcohol in saline, polyethylene glycol, and cottonseed oil. A single dose of the appropriate test article extract was injected into five mice per extract by either the intravenous or intraperitoneal route. Similarly, five mice were dosed with each corresponding blank vehicle.

The animals were observed immediately at 4, 24, 48, and 72 hours after systemic injection. If, during the observation period, none of the mice treated with the individual test extract exhibited a significantly greater reaction than the corresponding control mice, the test extract met the USP requirements. If two or more mice died, or if abnormal behavior such as convulsions, or prostration occurred in two or more mice, or if body weight loss greater than 2 grams occurred in three or more mice, the test sample did not meet the USP requirements. Under the conditions of this study, the test article extracts would not be considered systemically toxic to the mouse at the prescribed USP dosage. Each test extract met the USP requirements.

The MEM elution study for cytotoxicity was also conducted. A 52.8 square centimeter portion of the foam was used. A monolayer of L-929 mouse fibroblast cells is grown to confluency and exposed to an extract of the test article prepared by placing the foam in 18 ml of 5% Minimum Essential Medium and extracting at 37° C for 24 hours. Duplicate MEM aliquots were used as negative controls. The positive control was extracted at 37° C for 24 hours and tested using the end point titration procedure.

After exposure to the extracts the cells were examined microscopically for cytotoxic effect.

The response of the cell monolayer was evaluated and the subsequent biological reactivity rated on a scale of 0-4. The test article is considered suitable if no signs of cellular reactivity are noted for the negative control article, and the positive control article shows greater than mild reactivity (grade 2). The test article meets the requirements of the test if none of the cultures treated with the test article show greater than a mild reactivity (grade 2). Presence or absence of a confluent monolayer, vacuolization, cellular swelling, crenation, and the percentage of cellular lysis is recorded. The purpose of this test is to determine the biological reactivity of a mammalian cell culture in response to the test article. The study is conducted based on the procedural specifications described in the US Pharmacopeia 23, National Formulary 18, pp. 1697-1695, 1995. Results of this test were somewhat ambiguous. The initial test completed by NaMSA showed a toxic reaction within the first 24 hours of the test. The test indicated a 100% lysis of the confluent monolayer.

These results were not indicative of the previous results obtained through the USP Class VI tests and other cytotoxicity tests. The MEM Elution study was then repeated by NaMSA. The second MEM test showed the material to be non-toxic, clearly in conflict with the prior report. However, all prior data, save the original MEM test, shows the material to be in conformance and therefore biocompatible. Upon further review by the testing laboratories it is evident that the material, as a borderline non-toxic material, could conceivably flip one way or the other if the cell line which made up the second test were hardier than those of the original study. NAmSA concluded that the in vivo toxicological tests were acceptable and the product should not be threatened by the ambiguous cytotoxic results of the MEM assay.

It is also possible that autoclaving this material is not the preferred method of sterilization. If there is any breakdown of the material form the high temperature cycle, free polyol would be eluted from the test sample. The polyol itself is relatively non-toxic, but it is a detergent like material that could potentially effect the MEM test.

The Ames Mutagenicity tests are now complete. The Salmonella typhimurium Reverse Mutation Assay (Ames Assay) evaluates the potential of the polyurethane foam to induce histidine reversion (his^- to his^+), caused by base changes or frameshift mutations in the genome of this organism. This direct plate incorporation assay is conducted using four strains of Salmonella typhimuriam, in the presence and absence of an exogenous mammalian activation system.

Historically, the Reverse Mutation Assay in Salmonella typhimuriam has been used to detect mutation in a gene of a histidine requiring strain to produce a histidine independent strain of this organism. This test system is recommended in TSCA 40 CFR, part 798, Subpart F, section 798.5265, 1989; and Maron, D.M., Ames, B.N., 1983, revised methods for the Salmonella Mutagenicity Test, Mutation Research: 113. Pp. 173-215. Under the conditions of this assay, a saline extract of the foam carrier was not considered mutagenic to Salmonella typhimurim tester strains TA98, TA100, TA1535, TA1537, and TA1538.

The Kligman Maximization study has also been completed. The purpose of the Kligman Maximization study is to evaluate the allergenic potential or sensitizing capacity of the test article. The test is used as a procedure for screening of contact allergens in guinea pigs and extrapolating the results to humans, but it does not establish the actual risk to humans.

The test article itself is extracted and spread over a 2x4 cm piece of filter paper to saturation. The patch is then covered by an impermeable sheet and secured with a non adhesive bandage, which is wound around the torso of the animal. The dressing is left in place for 48 hours. Using the scoring system of Kligman, the allergenic potential of the test article is classified as Grade I (weak) to Grade V (extreme), using the percentage of sensitization observed.

The final toxicity test performed was the Rabbit Pyrogenicity Test. The pyrogen test is designed to limit to an acceptable level the risks of febrile reaction in the patient to the administration of the product concerned. The test involves measuring the rise in temperature of albino rabbits following the intravenous injection of a test article. It is designed for products that can be tolerated by the test rabbit in a dose not to exceed 10 ml per Kg, within a period of not more than 10 minutes.

The test or control article is injected into the marginal ear vein of each rabbit at a dose based on the body weight of each animal. Each injection must be completed within 10 minutes after the start of administration. Body temperature is recorded at 30 minute intervals between 1 and 3 hours subsequent to injection. All testing was conducted according to the requirements of USP 23.

With the exception of the somewhat ambiguous results obtained in the MEM elution tests, the results of all other tests were negative, and the sponge material appears to be suitable as a carrier for the tissue adhesive. The summary results and Class VI certification of the foam material from NAmSA are reproduced in Appendix B.

Conclusions

A schedule of the first year activities appears on a following page. The program is basically on schedule at this point, and no significant problems have yet been identified.

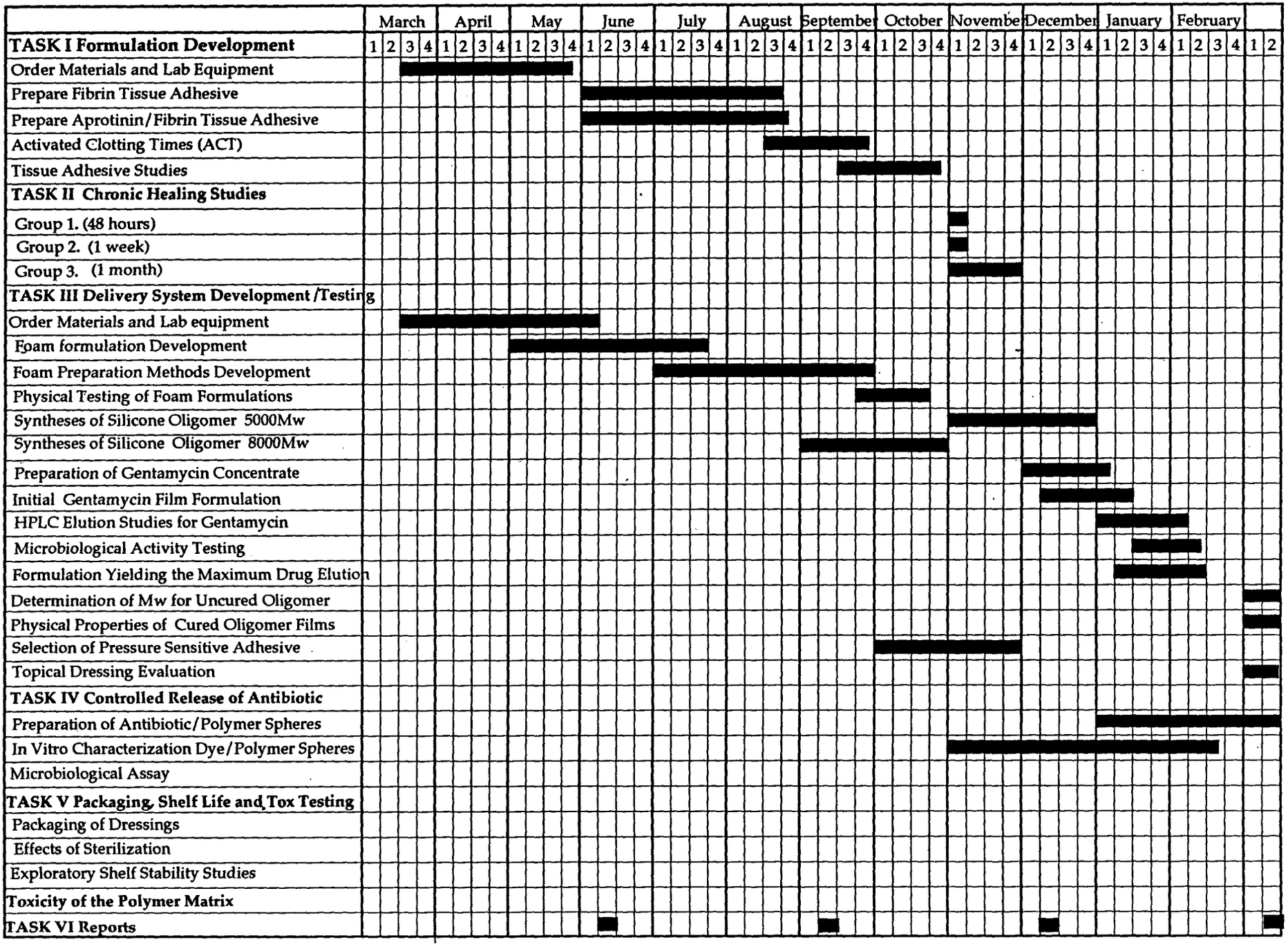
In the first year of this program, significant milestones were achieved. These included: (1) completion of the development of our manufacturing processes for the fibrin tissue adhesive: (2) the start of long term animal studies with the agent to determine its effects on wound healing; (3) the development of the final formulation for the hydrophilic polyurethane foam carrier for the tissue adhesive to control bleeding with deep tissue injury; (4) the development of a soft silicone cured film with the inclusion of antibiotics to be used in combination with the tissue adhesive to treat superficial injuries; and (5) the completion of toxicity testing of the hydrophilic polyurethane foam material.

The foam material passed all USP Class VI requirements and appears suitable for use in this application. U.S. Patent 5,464,471 (Appendix A) for the new tissue adhesive has also been issued. We believe this patent covers the basic formulation of the new tissue adhesive and both delivery systems which are now being developed.

Anticipated Activities

Work is ongoing in the development of the antimicrobial wound dressing to be used with the tissue adhesive for treating superficial injuries. Currently underway are peel strength tests, adhesive property tests, as well as drug elution studies to help determine the best antimicrobial for this device. In the next reporting period, the in vivo experimentation being carried out at the Cleveland Clinic Foundation to examine the effects of the agent on final wound healing will be continued. Those experiments were delayed to allow for completion of work on the foam material, which is to be evaluated acutely at the termination of these experiments. The remaining biocompatibility testing will also be completed, and testing on the antimicrobial laden films will be conducted.

Fibrin Tissue Adhesive Work Schedule Year 1 1995



Appendix A

U.S. Patent 5,464,471
Fibrin Monomer Based Tissue Adhesive

US005464471A

United States Patent [19]

Whalen et al.

[11] Patent Number: 5,464,471

[45] Date of Patent: Nov. 7, 1995

[54] **FIBRIN MONOMER BASED TISSUE ADHESIVE**

[75] Inventors: **Robert L. Whalen**, Somerville; **Donald Dempsey**, Newbury, both of Mass.

[73] Assignee: **Whalen Biomedical Inc.**, Somerville, Mass.

[21] Appl. No.: **339,176**

[22] Filed: **Nov. 10, 1994**

[51] **Int. Cl.**[6] **C09J 189/00**

[52] **U.S. Cl.** **106/124**; 604/290; 514/12

[58] **Field of Search** 106/124; 606/214; 604/290; 514/12

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,969,240	7/1976	Kolobow et al.	210/321
4,362,567	7/1982	Schwarz et al.	106/157
4,414,976	11/1983	Schwarz et al.	106/161
4,735,616	4/1988	Eibl et al.	606/191
4,909,251	3/1990	Seelich	606/213
5,219,895	6/1993	Kelman et al.	522/68
5,330,974	7/1994	Pines et al.	514/21

Primary Examiner—David Brunsman

[57] **ABSTRACT**

A single agent fibrin based hemostatic and tissue adhesive agent comprised of recombinant fibrin monomer, Bovine thrombin, and calcium chloride, which is lyophilized to powder form, and is activated upon blood contact, at the site of tissue injury, producing effective hemostasis and subsequent tissue adhesion.

7 Claims, No Drawings

FIBRIN MONOMER BASED TISSUE ADHESIVE

LICENSE RIGHTS

The U.S. government has a paid up license in this invention and the right, in limited circumstances to require the patent owner to license others on reasonable terms as provided by U.S. Army contract number DAMD 12-94-C-4039.

BACKGROUND

1. Field of the Invention

This invention relates to a fibrin based tissue adhesives for achieving hemostasis.

2. Description of Prior Art

The ability to establish expeditious and permanent bonding between biological tissues is a critical factor in the success of many medical procedures, from surgical operations to wound dressings. Attempts to provide such bonding through mechanical means have proven inadequate. Consequently, biomedical research has focused on the development of natural and synthetic materials to act as adhesives, sealants and hemostatic agents. In connection with the description of previous inventions and the invention herein, the terms adhesive, sealant and hemostatic agent are defined broadly and used as these terms are understood in the art.

The clinical applications of such tissue adhesive agents are extensive and diverse. Aside from simple hemorrhage control and wound closure, applications include the treatment and preservation of the ruptured spleen (Brands, W. et al., World J. Surg., 6. 366–368, (1982)), the sealing of vascular prostheses (Walterbusch. G. et al., Thorac. cardiovasc. Surgeon, 30, 234–235, (1982)), the sealing of vascular grafts prior to implantation (Kalmer, P. et al., Thorac. cardiovasc. Surgeon, 30, 230–23 1, (1982)), the sealing of microvascular anastomoses (Pearl, RM et al., Surgery, Gynecology & Obstetrics, 144, 227–230, (1977)), the repair of middle ear defects (Epstein, GH et al., Ann Otol. Rhinol. Laryngol., 95, 40–45, (1986), and Silberstein. LE et al., Transfusion, 28(4), 319–321, (1988)), and the bonding of a corneal inlay into a recess prepared to receive same in the cornea of a patient. In fact, marketing research has indicated that there are over 8 million surgical procedures that could utilize a safe, effective biological adhesive.

Major interest in the use of synthetic polymeric materials to act as adhesives, sealants and hemostatic agents began in the early sixties. Initial work was confined to water-soluble systems such as casein and polyvinyl alcohol, but was later expanded to include all available synthetic adhesives and plastics with no known local or general toxicity. Although many materials were investigated, the most widely used tissue adhesives were the cyanoacrylates. These are a homologous series of organic molecules which polymerize and adhere to moist living tissues. Methyl-alpha-cyanoacrylate (MCA) in particular, has been used since 1960 by many investigators as a non-suture adhesive for bones. MCA is a fluid, monomeric material which polymerizes in seconds under mild pressure to produce a thin, strong, adherent film. However, these materials have been shown to be histotoxic and induce detrimental inflammatory tissue reaction.

Such toxicity with synthetic adhesives has led investigations toward the development of biologically derived bonding materials. These materials often consist of elements from the natural bonding mechanism, such as collagen or fibrin. Collagen is a major connective tissue protein which is evident in prior art of many biomedical products, such as an artificial cornea (U.S. Pat. No. 4,581,0303, a hemostatic agent (U.S. Pat. No. 4,2 15,2003 and a soft contact lens (U.S. Pat. Nos. 4,264,155: 4,264,493; 4,349,470; 4,388,428; 4,452,925 and 4,650,6163, due to its good biocompatibility. However, natural collagen must be modified to render it suitable for use as a biomedical adhesive.

In many instances, the prior modified collagen-based adhesives suffer from various deficiencies which include (1) crosslinking/polymerization reactions that generate exothermic heat, (2) long reaction times, and (3) reactions that are inoperative in the presence of oxygen and physiological pH ranges (Lee, ML et al., Adhesion in Biological Systems, RS Manly, ed., Academic Press, New York, 1970. Ch. 17) Moreover, many of these adhesives contain toxic materials rendering them unsuitable for biomedical use. As a result, recent processing developments have been revealed, as in U.S. Pat. No. 5,219,895 to Kelman, in which pure, soluble or partially fibrillar collagen monomers are chemically modified to be soluble at physiological conditions and polymerize to achieve sealant properties.

Improvements of this sort still have not produced an agent as effective in achieving hemostasis as fibrin based tissue adhesives. Compared with oxidized cellulose, microfibrillar collagen, or surface charge modified collagen, the use of fibrin adhesive results in significantly less blood loss at the site of injury (Raccuia, JS et al. Comparative efficacy of topical hemostatic agents in a rate kidney model. Am. J. Surg. 163(2):234–8, 1992). Consequently, tissue adhesives of this type have been developed as seen in U.S. Pat. Nos. 4,362,567 and 4,414,976 and Can. Pat. No. 1,168,982. The foundation of these agents are the proteins fibrinogen and thrombin.

Fibrinogen is a soluble protein found in the blood plasma of all vertebrates. When fibrinogen is contacted by thrombin, a protein enzyme, it is converted into fibrin monomer and Factor XIII is activated to Factor XIIIa. Factor XIIIa then polymerizes the fibrin monomer to form a stabilized fibrin network. Such a network is essential to the healing process of wound closure and tissue bonding. The network serves as a physical bond and as a scaffolding to support the migration of immunologically active cells, for defense against invading pathogens, and epithelial cells, for tissue regeneration and repair. The fibrin network may then be gradually dissolved by the body (fibrinolysis) after treatment leading to a more normal appearance of the healed site.

Tissue adhesive preparations of this type usually consist of a fibrinogen solution containing Factor XIII, some additional proteins, such as fibronectin and albumin, and active or nonactive additions. A thrombin solution may also be provided containing thrombin and calcium ions, or the thrombin may be provided from the tissue area to be bonded itself. These solutions are commercially available in the form of either deep-frozen solutions or lyophilisate due to their lack of stability as liquid aqueous solutions. Therefore, these products are typically packaged in the form of kits, which include the protein ingredients, means to prepare the solutions, and means to utilize the solutions.

In emergency situations, quick availability of the tissue adhesive may be of decisive importance. However, the use of these kits is often difficult, tedious and time consuming. These constraints pose a problem in the hospital setting and may be completely defeating in the field or combat settings. To overcome this, there have been attempts to shorten the

preparation time. This usually involves shortening the reconstitution time of lyophilized solutions. For instance, Can. Pat. No. 1,182,444 describes a method and an arrangement for accelerating the dissolution of lyophilized medicines. The combined heating and stirring device disclosed markedly shortened reconstitution times, yet physicians have voiced a desire for further improvements.

It has been known that the solubility of hard-soluble proteins can be improved by certain additions. Thus, EP-A-0 085 923 discloses a lyophilized fibrinogen composition which additionally contains a further substance having a urea or guanidine residue. However, it has been shown that such additions have a cytotoxic effect, inhibit the growth of fibroblasts and cause the formation of an irregular fibrin structure resulting in the loss of desired elasticity of the fibrin. These effects jeopardize the desired properties of fibrinogen-based tissue adhesives, such as the stimulation of wound healing and the capacity for high strain.

Others, such as U.S. Pat. No. 4,909,251 to Seelich, utilize a biologically compatible tenside addition to the fibrinogen composition, and optionally further proteins as well as adjuvants or additives, to reduce reconstitution times. The tenside is from the group of non-ionic, cationic, anionic or zwitterionic tensides and is present in an amount from 0.03 to 15% by mass based on the fibrinogen content. Additions such as this have been shown to be useful in preparations having a high content of fibronectin, a plasma protein which is difficult to dissolve.

However, there are still existing inherent disadvantages to the present system of hemostatic and tissue adhesive compounds. The therapeutic compositions of some fibrin sealants and agents still contain non-autologous, non-single donor human fibrinogen, that is they comprise fibrinogen derived, or pooled from multiple human donors. Because of the risk of viral disease such as AIDS, hepatitis B and C, these compositions are not in use in the United States. With various incidents of infection reported it is unlikely that these compounds would ever be released for use in the United States.

Accordingly, practitioners of the art have sought to provide autologous or single donor fibrinogen compositions to minimize the risk of viral infection. However, substantial variation in the fibrinogen content of such preparations has lead to difficulty in predicting, accurately, the clinically effective dose required.

An alternate resolution to the above mentioned risks characteristic of human plasma derived therapeutic products was to provide fibrinogen from a mammalian source other than humans. This, however, can result in a severe immune response. Even the currently available highly purified bovine fibrinogen compositions, such as those indicated in U.S. Pat. No. 5,330,974, contain some foreign antigen.

A significant improvement in the design of fibrin tissue adhesives would involve the complete elimination of such solution preparation and mixing time, eliminate the risk of viral transmission, and severe immunologic response. A tissue adhesive of this type would consist of a blood activated single agent that is usable in a dry form, utilizing genetically engineered fibrin monomer, thus avoiding the time and constraints of preparing and pre-mixing ingredients, as well as any risk of viral transmission. The present invention provides such an agent. The new agent is distinguished from previously described fibrin tissue adhesives in that it is prepared using a genetically engineered fibrin monomer rather than fibrinogen. This allows it to be lyophilized from a single solution containing all of its constituents, including thrombin. Consequently, the agent is available as a dry powder which is activated upon blood contact producing effective hemostasis and subsequent adhesion.

Numerous publications describe the successful use of a simplified fibrin glue consisting of fibrinogen, thrombin, calcium chloride and Factor XIII (Kjaergard, HK et al., Ann Thorac Surg, 55(2):543–44, 1993; Hartman, AR et al., Arch Surg, 127:357–59, 1992; Dahlstrom, KK et al., Plastic and Reconstructive Surgery, 89(5):969–976, 1992). If one assumes that sufficient Factor XIII will be present in the blood being lost at the site of injury, it is possible to prepare a fibrin glue for hemorrhage control consisting of fibrinogen, thrombin and calcium chloride. One problem in preparing a solution with these ingredients, however, is the formation of a fibrin gel directly from the interaction of fibrinogen and thrombin. Therefore, a solution containing fibrin monomer is used instead of its precursor fibrinogen.

In the early stages of polymerization, the fibrin monomer molecules attach to each other by loose hydrogen and hydrophobic bonds which can be broken apart with ease. It is at this point that the fibrin monomer is utilized. The loosely bound monomers are chemically disrupted and the mixture is sonicated or shaken until the gel is dissolved. The dissolved fibrin monomer is then used to prepare a solution with thrombin and calcium chloride. This solution is finally lyophilized to dryness to provide a single agent fibrin tissue adhesive.

This method of preparation is necessary because it is not feasible to simply mix separately lyophilized powders in the correct proportions. The principal difficulty is in uniformly mixing the ingredients. The amount of thrombin, for example, is quite small compared to the amount of clottable protein: thus, it would be extremely difficult to thoroughly blend the two powders to achieve a consistent mixture. The method of the inventors produces a uniform preparation by virtue of being lyophilized from a true solution of its constituents.

When the lyophilized agent is exposed to Factor XIII, present in the blood at the site of use, the fibrin monomer will precipitate as stabilized fibrin with the appropriate adhesive qualities. The added thrombin, of course, also induces platelet aggregation which assists in achieving hemostasis.

The formulation of the single agent tissue adhesive may have additions, such as aprotinin, an inhibitor of fibrinolysis, or antibiotics. Likewise, numerous "inert" additives (substances such as preservatives, dispersants or additional diluents) known in the art can be added to the therapeutic compositions of the invention, with the understanding that such substances are physiologically compatible.

The therapeutic compositions and methods defined by the present invention are useful in connection with any of the clinical applications where adhesives. sealants and hemostatic agents can be used. Tissue adhesion, sealing of tissue or hemostasis are induced in a mammalian patient at a site of treatment by contacting the treatment site with a therapeutically effective amount of composition. According to the practice of the invention, such effective amounts need not be equivalent to amounts that cause complete or permanent adhesion of tissue, causing total sealing of tissue boundary or complete arrest of bleeding or loss of tissue fluid from a tissue or tissue boundary. Rather, such compositions are within the scope of the invention if the use thereof provides at least a partial effect that is of benefit to the patient in the course of treatment.

Amount of agent necessary to perform clinical procedures

varies widely depending on, for example, the size of the treatment site, the nature of the condition in need of treatment and factors unique to each patient. It is accepted in the art that it is the skill of the clinical practitioners to determine for each patient and for each condition the amounts of the agent that are effective.

The lyophilized composition of the invention may be used directly in powder form by directly sprinkling the agent onto a wound site or surgical incision. As it reacts with the blood and tissue fluids at the site, it will effect a seal or hemostasis. This is typically useful when the site to be closed is small and blood loss is not rapid.

Additionally, the lyophilized composition may be applied to a wound or surgical incision by, for example, incorporation into a gauze pad, sponge, collagen or gel-type matrix or into a similar device in treating the area. This is useful in controlling bleeding due to deep tissue injury involving arterial blood loss. It may also be useful in the treatment of more superficial wounds in which external semi-occlusive dressings may be applied.

SUMMARY OF THE INVENTION

With the above in view, it is therefore among the primary objectives of this invention to provide a single agent fibrin based hemostatic and tissue adhesive agent comprised of recombinant fibrin monomer, Bovine thrombin, and Calcium Chloride, which is lyophilized to powder form, and is activated upon blood contact, at the site of tissue injury, producing effective hemostasis and subsequent tissue adhesion.

It is another object of the present invention to provide a hemostatic agent/tissue adhesive which utilizes a recombinant fibrin monomer thus eliminating the risk of pooled human plasma derived fibrin, as well as eliminating severe immunologic reactions to pooled mammalian derived fibrin.

It is still another object of the invention to provide a fibrin monomer hemostatic agent/tissue adhesive which can be applied directly to the site of injury, in powdered form, without reconstitution.

It is yet another object of the invention to provide a therapeutic single agent fibrin monomer tissue adhesive, tissue sealant, or hemostatic agent derived from the thrombin-fibrinogen polymerization reaction.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides for a lyophilized fibrin monomer containing therapeutic composition effective as a tissue adhesive, tissue sealant, or hemostatic agent. A therapeutic single agent fibrin monomer tissue adhesive is derived from the thrombin-fibrinogen polymerization reaction, which is then prevented from stabilizing.

Due to the structure of the coagulation cascade, the fibrin monomer may be obtained in vitro by a variety of approaches. First, it is known that fibrin monomer is formed when the protein enzyme, thrombin, acts on the protein fibrinogen. With its proteolytic capabilities, thrombin removes two low molecular weight peptides from each molecule of fibrinogen, forming a molecule of fibrin monomer. This monomer has the automatic capability of polymerizing with other fibrin monomer molecules, forming long fibrin threads within seconds. Unlike other tissue adhesive and hemostatic agents which rely on fibrinogen, the present invention makes use of the fibrin monomer, a totally separate molecule.

In the early stages of this polymerization, the fibrin monomer molecules attach to each other by loose hydrogen and hydrophobic bonds which can be broken apart with ease. It is at this point that the fibrin monomer may be utilized. However, under normal conditions, the blood plasma of vertebrates contains the inactive precursor of an enzyme, factor XIII, which can stabilize or strengthen the fibrin gels by introducing covalent bonds between the monomers. Activated Factor XIII binds neighboring molecules by covalently joining the side chains of certain glutamine acceptors. As a result of this cross-linking, stabilized fibrin has somewhat different properties than fibrin monomers.

As the present invention employs fibrin monomer, as opposed to fibrinogen, which all other tissue adhesives and hemostatic agents employ, the problems inherent in a fibrinogen system are eliminated. The problems in using fibrinogen, in a lyophilized formula, include the formation of a fibrin gel in the solution prior to lyophyllization because of the presence of thrombin. Thus lyophilizing a solution containing fibrin monomer instead of its precursor, fibrinogen, provides a solution to the problem of a single agent tissue adhesive, hemostatic agent. This solution lyophilizes to yield a truly homogeneous powder. When exposed to blood, thrombin activation of Factor XIII will precipitate the dissolved fibrin monomer as insoluble fibrin. The added thrombin also induces platelet aggregation which assists in achieving hemostasis.

The way in which fibrin monomer is derived from the thrombin-fibrinogen polymerization reaction and prevented from stabilizing is done at a point in which the fibrin monomer is easily disrupted during the reaction.

Lyophilized fibrinogen and thrombin are combined in a water solution. The fibrinogen is first dissolved at room temperature in distilled water and then subjected to sonication. Thrombin was then reconstituted, per directions. After dissolution, thrombin solution is injected into the fibrinogen solution.

Polymerization ensues instantaneously to form a soft gel. At this point the fibrin monomers are loosely bound and can be disrupted easily. To accomplish this disruption, a 10% ammonium hydroxide solution is added to the above solution, and the mixture is then sonicated or agitated until the gel is dissolved. The resultant fibrin monomer solution is then ready to be mixed into a final solution containing both thrombin and calcium chloride. This allows the solution to be lyophilized from a single solution containing all of its constituents, including thrombin. The agent is thus composed of fibrin monomer, thrombin, and calcium chloride; while it is assumed that sufficient factor XIII is present in the blood being lost at the site of injury.

The present invention relies on the presence of Factor XIII in the patient's own blood. When exposed to Factor XIII, the lyophilized fibrin monomer combination will precipitate as insoluble fibrin. The present invention therefore provides for a blood activated system employed as a powder without the premixing of ingredients.

What is claimed is:

1. A single agent hemostatic tissue adhesive comprising a lyophilized powder of fibrin monomer from human recombinant fibrinogen, calcium chloride, and bovine thrombin.

2. The invention of claim **1** whereto said hemostatic tissue adhesive does not require preparation, additives, or time, prior to use.

3. The invention of claim **1** consisting essentially of a lyophilized powder of fibrin monomer from human recom-

binant fibrinogen, calcium chloride, and bovine thrombin.

4. The invention of claim **1** wherein said fibrin monomer is from a human recombinant fibrinogen source free of virus.

5. A method of eliminating the risk of immunologic reaction to fibrinogen comprising applying the composition of claim **4** to injured tissue.

6. A method of eliminating the risk of immunologic reaction to fibrinogen comprising applying the composition of claim **1** to injured tissue.

7. A method of using a hemostatic tissue adhesive comprising using the invention of claim **1** with gauze, sponges, or other wound dressings in combination.

* * * * *

Appendix B

Toxicity Test Results for the Hydrophilic Polyurethane Foam Material

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CERTIFICATE OF COMPLIANCE
USP BIOLOGICAL TESTS

CLASSIFICATION VI

Test Article: FOAM

ACUTE SYSTEMIC TOXICITY (USP): Test article extracts in saline, alcohol in saline, polyethylene glycol 400 and cottonseed oil did not produce a significantly greater systemic reaction than the blank extractant when injected into mice.

INTRACUTANEOUS TOXICITY (USP): Test article extracts in saline, alcohol in saline, polyethylene glycol 400 and cottonseed oil did not produce a significantly greater tissue reaction than the blank extractant when injected intracutaneously into rabbits.

IMPLANTATION TEST (USP): The macroscopic reaction of the test article, implanted in rabbit muscle for 5 days, was not significant when compared to the USP negative control plastic.

The sample of test article extracted at a ratio of 60 cm^2:20 ml and at a temperature of 70°C for 24 hours, met the requirements of a USP Class VI Plastic.

las Completed 12-18-95 Approved T. J.

MMQ

TU006-600

TABLE I

USP SYSTEMIC TOXICITY OBSERVATIONS

MORTALITY AND BODY WEIGHT DATA:

	TEST EXTRACT				CONTROL BLANK			
Extract, Route and Dose	Animal Number	Weight (g) Day 0	Weight (g) Day 3	#Dead/ #Tested	Animal Number	Weight (g) Day 0	Weight (g) Day 3	#Dead/ #Tested
Sodium Chloride Injection (SC) IV; 50 ml/kg	1	17	19	0/5	1	18	19	0/5
	2	17	20		2	18	20	
	3	18	21		3	17	17	
	4	17	21		4	18	21	
	5	17	20		5	17	18	
Alcohol in Sodium Chloride 1:20 (AS) IV; 50 ml/kg	1	19	19	0/5	1	17	17	0/5
	2	20	21		2	17	17	
	3	18	18		3	17	19	
	4	20	20		4	17	20	
	5	18	19		5	18	19	
Polyethylene Glycol 400 (PEG) IP; 10 g/kg	1	17	18	0/5	1	18	17	0/5
	2	19	21		2	18	18	
	3	18	18		3	17	18	
	4	19	19		4	17	18	
	5	20	19		5	17	17	
Cottonseed Oil (CSO) IP; 50 ml/kg	1	18	17	0/5	1	18	21	0/5
	2	18	19		2	17	20	
	3	17	16		3	18	19	
	4	17	18		4	17	19	
	5	19	20		5	17	19	

CLINICAL OBSERVATIONS:

	TEST EXTRACT				CONTROL BLANK			
	SC	AS	PEG	CSO	SC	AS	PEG	CSO
Immediate	AN	L	AN	AN	AN	L	AN	AN
4 Hours	AN	AN	AN	AN	AN	AN	AN	AN
24 Hours	AN	AN	AN	AN	AN	AN	AN	AN
48 Hours	AN	AN	AN	AN	AN	AN	AN	AN
72 Hours	AN	AN	AN	AN	AN	AN	AN	AN

AN = Appeared Normal
L = Lethargic

TABLE I

USP INTRACUTANEOUS TOXICITY OBSERVATIONS

RABBIT NUMBER	EXTRACT	TEST EXTRACT						CONTROL BLANK					
		Scoring Interval (HOURS)						Scoring Interval (HOURS)					
		24		48		72		24		48		72	
		ER	ED	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED
91622	SC	0	0	0	0	0	0	0	0	0	0	0	0
91624	SC	0	0	0	0	0	0	0	0	0	0	0	0
91622	AS	0	0	0	0	0	0	0	0	0	0	0	0
91624	AS	0	0	0	0	0	0	0	0	0	0	0	0
91625	PEG	0	0	0	0	0	0	0	0	0	0	0	0
91638	PEG	0	0	0	0	0	0	0	0	0	0	0	0
91625	CSO	1	1	1	1	1	1	2	2	2	2	1	1
91638	CSO	1	1	1	1	1	1	2	1	1	1	1	1

ER = Erythema
ED = Edema

SC = 0.9% sodium chloride USP solution
AS = alcohol in saline (1:20)
PEG = polyethylene glycol 400
CSO = cottonseed oil, NF

TABLE I

USP INTRACUTANEOUS TOXICITY OBSERVATIONS

RABBIT NUMBER	EXTRACT	TEST EXTRACT						CONTROL BLANK					
		Scoring Interval (HOURS)						Scoring Interval (HOURS)					
		24		48		72		24		48		72	
		ER	ED	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED
91622	SC	0	0	0	0	0	0	0	0	0	0	0	0
91624	SC	0	0	0	0	0	0	0	0	0	0	0	0
91622	AS	0	0	0	0	0	0	0	0	0	0	0	0
91624	AS	0	0	0	0	0	0	0	0	0	0	0	0
91625	PEG	0	0	0	0	0	0	0	0	0	0	0	0
91638	PEG	0	0	0	0	0	0	0	0	0	0	0	0
91625	CSO	1	1	1	1	1	1	2	2	2	2	1	1
91638	CSO	1	1	1	1	1	1	2	1	1	1	1	1

ER = Erythema
ED = Edema

SC = 0.9% sodium chloride USP solution
AS = alcohol in saline (1:20)
PEG = polyethylene glycol 400
CSO = cottonseed oil, NF

TABLE 1

USP SYSTEMIC TOXICITY OBSERVATIONS

MORTALITY AND BODY WEIGHT DATA:

	TEST EXTRACT				CONTROL BLANK			
Extract, Route and Dose	Animal Number	Weight (g) Day 0	Weight (g) Day 3	#Dead/ #Tested	Animal Number	Weight (g) Day 0	Weight (g) Day 3	#Dead/ #Tested
Sodium Chloride Injection (SC) IV: 50 ml/kg	1	17	19	0/5	1	18	19	0/5
	2	17	20		2	18	20	
	3	18	21		3	17	17	
	4	17	21		4	18	21	
	5	17	20		5	17	18	
Alcohol in Sodium Chloride 1:20 (AS) IV: 50 ml/kg	1	19	19	0/5	1	17	17	0/5
	2	20	21		2	17	17	
	3	18	18		3	17	19	
	4	20	20		4	17	20	
	5	18	19		5	18	19	
Polyethylene Glycol 400 (PEG) IP: 10 g/kg	1	17	18	0/5	1	18	17	0/5
	2	19	21		2	18	18	
	3	18	18		3	17	18	
	4	19	19		4	17	18	
	5	20	19		5	17	17	
Cottonseed Oil (CSO) IP: 50 ml/kg	1	18	17	0/5	1	18	21	0/5
	2	18	19		2	17	20	
	3	17	16		3	18	19	
	4	17	18		4	17	19	
	5	19	20		5	17	19	

CLINICAL OBSERVATIONS:

	TEST EXTRACT				CONTROL BLANK			
	SC	AS	PEG	CSO	SC	AS	PEG	CSO
Immediate	AN	L	AN	AN	AN	L	AN	AN
4 Hours	AN	AN	AN	AN	AN	AN	AN	AN
24 Hours	AN	AN	AN	AN	AN	AN	AN	AN
48 Hours	AN	AN	AN	AN	AN	AN	AN	AN
72 Hours	AN	AN	AN	AN	AN	AN	AN	AN

AN = Appeared Normal
L = Lethargic

Results and conclusions apply only to the test article tested. No further evaluation of these results is made by NAmSA. Any extrapolation of these data to other samples is the responsibility of the sponsor.

RESULTS

Clinical Observations: All animals appeared clinically normal throughout the duration of the study. Weight gain for individual rabbits was considered acceptable.

Macroscopic Observations: The findings for the macroscopic evaluation are shown below.

Rabbit Number	Weight (kg)		Site	Test	USP Negative Control
	Initial	Day 5			
91571	2.6	2.8	1 2 3 4	0 0 0 0	0 0 0
91573	2.6	2.6	1 2 3 4	0 0 0 0	0 0 0
			Average:	0.0	0.0

Macroscopically, there was no difference between the reactions of the test and control materials. This resulted in a macroscopic reaction of "not significant" tissue contact irritation.

CONCLUSION

Under the conditions of this study, the macroscopic reaction was not significant as compared to the USP negative control implant material. The implanted test article met the USP requirements.

RECORD STORAGE

All raw data pertaining to this study and a copy of the final report are to be retained in designated NAmSA archive files.

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Table I

STRAIN CHARACTERISTICS AND STANDARD PLATE COUNTS

Characteristics (expected)	Tester Strains				
	TA98*	TA100*	TA1535†	TA1537*	TA1538*
Ampicillin-TA98 & TA100 = (Resistant) TA1535, TA1537 & TA1538 = (Sensitive)	R	R	S	S	S
Rfa Mutation; CV (Sensitive)	S	S	S	S	S
UVrb (No Growth)	NG	NG	NG	NG	NG
Histidine Requirement; (Growth)	G	G	G	G	G
Biotin (No Growth)	NG	NG	NG	NG	NG
Purity (Pure)	PURE	PURE	PURE	PURE	PURE
Total Plate Count Plate CFU's (10^{-7})	303 325 314	302 332 317	329 364 347	222 280 251	321 275 298
Titer (Organisms/ml)	3.1×10^9	3.2×10^9	3.5×10^9	2.5×10^9	3.0×10^9

R = Resistant S = Sensitive NG = No Growth G = Growth N/A = Not Applicable
*Tester strain TA100 2-aminofluorene with and without S-9 was repeated at the same time that TA1535 was repeated to verify the activity of the rat liver preparation (S-9). Plate counts obtained were acceptable. Values presented are those of the preliminary test.
†Tester strain TA1535 was out of the expected spontaneous revertant range. Values presented are those of the retest.

Table II

SPOT PLATE INHIBITION SCREEN RESULTS

	Zone of Inhibition (mm)				
	TA98	TA100	TA1535	TA1537	TA1538
0.9% Saline (- control)	0	0	0*	0	0
0.9% saline test article extract	0	0	0*	0	0
Dexon (+ control)	37	31	30*	34	38

*Tester strain TA1535 was out of the expected spontaneous revertant range and was repeated. Values presented are those of the retest.

Table III

STANDARD PLATE INCORPORATION ASSAY RESULTS

	Salmonella typhimurium Tester Strains									
	TA98		TA100		TA1535*		TA1537		TA1538	
	CFTP	$\bar{x}$	CFTP	$\bar{x}$	CFTP	$\bar{x}$	CFTP	$\bar{x}$	CFTP	$\bar{x}$
0.9% saline (- control)	26 17 23	22	121 126 120	122	19 14 13	15	6 7 7	7	10 6 7	8
0.9% saline test article extract	22 17 17	19	151 148 129	143	33 21 24	26	5 6 8	6	8 7 6	7
0.9% saline w/S-9 (- control)	18 16 25	20	122 131 119	124	17 22 22	20	4 2 5	4	7 8 7	7
0.9% saline test article extract w/S-9	15 19 21	18	124 123 128	125	17 27 30	25	3 4 3	3	7 9 6	7
Dexon (+ control)	1664 1664 1840	1723	1072 1008 1344	1141	NA	NA	1024 1120 1248	1131	NA	NA
Dexon w/S-9 (+ control)	1616 1264 1088	1323	832 944 848	875	NA	NA	608 496 416	507	NA	NA
Sodium azide (+ control)	NA	NA	NA	NA	1280 1632 1296	1403	NA	NA	NA	NA
Sodium azide w/S-9 (+ control)	NA	NA	NA	NA	1664 1280 1632	1525	NA	NA	NA	NA
2-nitrofluorene (+ control)	NA	NA	NA	NA	NA	NA	NA	NA	2416 3152 2896	2821
2-nitrofluorene w/S-9 (+ control)	NA	NA	NA	NA	NA	NA	NA	NA	1280 1232 1488	1333
2-aminofluorene (+ control) (- control for S-9)	NA	NA	136† 122† 122†	127†	NA	NA	NA	NA	34 34 30	33
2-aminofluorene w/S-9 (+ control) (+ control for S-9)	NA	NA	1184† 1152† 1392†	1243†	NA	NA	NA	NA	1664 1504 1488	1552

CFTP = Counts from triplicate plates
$\bar{x}$ = Mean of triplicate plates
NA = Not Applicable

†Tester strain TA100 positive control (2-aminofluorene) with and without S-9 was repeated at the same time that tester strain TA1535 was repeated to verify the activity of the rat liver preparation (S-9). Plate counts obtained wei acceptable. Values presented are those of the preliminary test.
*Tester strain TA1535 was found to be out of the expected spontaneous revertant range and was repeated. Values presented are those of the retest.

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LAB NO. 95T 18466 01
P.O. NO. 1265

WHALEN BIOMEDICAL
LABORATORIES
11 MILLER STREET
SOMERVILLE, MA 02143-2918
ATTN: MICHAEL J. SARRASIN

ID NO. NOT SUPPLIED

CYTOTOXICITY - MEM ELUTION - MG023

Test Article: FOAM

Test Article Size Used: 53.5 sq. cm, 1 gram

Procedure: A monolayer of L-929 mouse fibroblast cells was grown to confluency and exposed to an extract of the test article prepared by placing the test article in 18 ml of 5% Minimum Essential Medium and extracting at 37°C for 24 hours. Duplicate MEM aliquots were used as negative controls. The positive control was extracted at 37°C for 24 hours and tested using an end-point titration procedure. After exposure to the extracts, the cells were examined microscopically for cytotoxic effect (CTE). Presence (+) or absence (-) of a confluent monolayer, vacuolization, cellular swelling and crenation and the percentage of cellular lysis were recorded.

CTE Score	Macroscopic Appearance of Cells
Nontoxic (N)	A uniform, confluent monolayer, with primarily elongated cells, and discrete intracytoplasmic granules present at the 24 hour observation. At the 48 and 72 hour observation periods, there should be an increasing number of rounded cells as cell population increases and crowding begins. Slight or no vacuolization, crenation or swelling should be present.
Intermediate (I)	Cells may show marked vacuolization, crenation or swelling. Cytolysis (0-50%) of cells that results in floating cells and debris in the medium may be present. The remaining cells are still attached to the flask surface.
Toxic (T)	Extensive vacuolization, swelling, or crenation are usually present in the cells remaining on the flask surface.

Results and conclusions apply only to the test article tested. No further evaluation of these results is made by NAmSA. Any extrapolation of these data to other samples is the responsibility of the sponsor.

las Completed 1-9-96 Approved Barry Phelps, BS

MG023-100

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LAB NO. 95T 18466 01

CYTOTOXICITY - MEM ELUTION - MG023

Results:	Confluent Monolayer	Vacuolization	Swelling	Crenation	% Lysis	CTE Score
24 HOURS						
Test Medium	(+)	(-)	(-)	(-)	0	N
48 HOURS						
Test Medium	(+)	(-)	(-)	(-)	0	N
72 HOURS						
Test Medium	(+)	(-)	(-)	(-)	0	N

Extract Conditions: Test - clear Controls - clear

Positive control, SCG-12, was toxic at a dilution of 1:8 at 24 hours.

The negative controls were acceptable (nontoxic) under these extraction conditions.

Conclusion: Nontoxic

Test Article Received: 1-02-96
Date Prepared: 1-4-96 Date Terminated: 1-8-96

Comments: The test medium was similar to the control medium at 24, 48 and 72 hours.
All raw data pertaining to this study and a copy of the final report are to be retained in designated NAmSA archive files.

las Completed_____ Approved_____ (See page 1)_____

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LAB NO. 96C 01629 00

CYTOTOXICITY - MEM ELUTION - MG023

Results:	Confluent Monolayer	Vacuolization	Swelling	Crenation	% Lysis	CTE Score
24 HOURS						
Test Medium	(-)	(-)	(-)	(-)	100	T
48 HOURS						
Test Medium			Not Applicable			
72 HOURS						
Test Medium			Not Applicable			

Extract Conditions: Test - Clear Controls - Clear

Positive control, SCG #3, was toxic at a dilution of 1:16 at 24 hours.

The negative controls were acceptable (nontoxic) under these extraction conditions.

Conclusion: (Toxic)

Test Article Received: 1-26-96
Date Prepared: 1-26-96 Date Terminated: 1-28-96

Comments: Test medium was more alkaline than the control medium at 24 hours.
All raw data pertaining to this study and a copy of the final report are to be retained in designated NAmSA archive files.

cb Completed ___---___ Approved ___(See page 1)___

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 19 Apr 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for Accession Document Numbers ADB210895, ADB223532, ADB209674, ADB231094, and ADB249633, be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at Virginia.Miller@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management